

ISOLATION AND CHARACTERIZATION OF KDOP SYNTHASE AND TWO  
ISOZYMES OF DAHP SYNTHASE IN Spinacia oleracea L. AND  
Solanum tuberosum L.

By

RON LOU DOONG

A DISSERTATION PRESENTED TO THE  
GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN  
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1990

Copyright 1990

by

Ron Lou Doong

## ACKNOWLEDGEMENTS

I offer my sincere gratitude to Dr. Roy Jensen who has provided financial support, help, and guidance throughout my endeavor to accomplish my goal in science. I am very much indebted to Dr. John Gander for his invaluable instructions on carbohydrate chemistry. Special thanks are also due to Dr. K. T. Shanmugam, Dr. P. Chun, Dr. D. Duggan, and Dr. W. Gurley for their advice while serving on my advisory committee.

I also wish to thank Dr. Randy Fischer, Dr. Tianhui Xia, Ms. Carol Bonner, and Mrs. Premila Rao for their friendly help and my fellow graduate students Prem Subramaniam and Genshi Zhao for the sharing of their wisdom. I owe my son Larry, my friend Ms. Eunice Johnson, Mr. Chris Fredette, Mr. Yaw-Hwa Liou and Dr. Simon Wang very much for their patient help with the proofreading and preparation of this dissertation.

## TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS.....	iii
LIST OF FIGURES.....	v
LIST OF TABLES.....	vi
ABSTRACT.....	vii
 CHAPTER	
I    LITERATURE REVIEW AND RATIONALE.....	1
II   DAHP SYNTHASE-Mn (DS-Mn).....	6
INTRODUCTION.....	6
MATERIALS AND METHODS.....	7
RESULTS.....	10
III  DAHP SYNTHASE-Co (DS-Co).....	44
INTRODUCTION.....	44
MATERIALS AND METHODS.....	44
RESULTS.....	46
DISCUSSION.....	59
IV   KDOP SYNTHASE.....	66
INTRODUCTION.....	66
MATERIALS AND METHODS.....	67
RESULTS.....	69
DISCUSSION.....	85
REFERENCES.....	86
BIOGRAPHICAL SKETCH.....	93

## LIST OF FIGURES

	<u>Page</u>
2-1 Elution profiles of potato DS-Mn, DS-Co, and KDOP synthase from DEAE cellulose.....	13
2-2 Elution profiles of spinach DS-Mn, DS-Co, and KDOP synthase from DEAE cellulose.....	15
2-3 pH optima of DS-Mn and DS-Co from spinach.....	19
2-4 Temperature optima of DS-Mn and DS-Co from spinach.	21
2-5 Divalent metal effects on spinach DS-Mn.....	24
2-6 Effects of DTT and thioredoxin on spinach DS-Mn....	26
2-7 Progress curve of fully activated spinach DS-Mn....	28
2-8 Saturation curve of spinach DS-Mn by PEP.....	34
2-9 Saturation curve of spinach DS-Mn by E4P.....	36
2-10 Inhibiton curve of spinach DS-Mn by arogenate.....	38
2-11 Induction of DS-Mn and DS-Co in potato tubers by mechanical wounding.....	40
3-1 Divalent metal effects on spinach DS-Co.....	51
3-2 Saturation curves of spinach DS-Co by PEP with various cosubstrates.....	53
3-3 Kinetics of periodate oxidation of enzymatic products of spinach DS-Co with various substrates..	57
4-1 Temperature optimum of spinach KDOP synthase.....	77
4-2 pH optimum of KDOP synthase from spinach.....	79
4-3 Thin-layer chromatography of KDOP and KDO.....	80
4-4 Kinetics of oxidation of KDOP by periodate.....	82
4-5 Double reciprocal plot for arabinose-5-phosphate...	84

## LIST OF TABLES

		<u>Page</u>
2-1	Thermostability of spinach DS-Mn.....	16
2-2	Effect of DTT and/or thioredoxin on DS-Mn activity from crude extracts of potato tubers and spinach leaves.....	29
2-3	Effect of $\beta$ -mercaptoethanol on the extraction of DS-Mn in potato tubers and spinach leaves.....	30
2-4	Effects of aromatic amino acids and intermediary metabolites on spinach DS-Mn at different pH's.....	31
3-1	Thermostability of spinach DS-Co.....	48
3-2	Relative velocity of spinach DS-Co with various substrates.....	54
3-3	Kinetic parameters for various substrates of spinach DS-Co.....	58
3-4	Relative configurations of hydroxy groups at C4 and C5 of enzymatic products of spinach DS-Co with various substrates as inferred from periodate oxidation kinetics.....	59
4-1	Thermostability of spinach KDOP synthase.....	74
4-2	General presence of KDOP snythase in higher plants.....	75

Abstract of Dissertation Presented to the Graduate School of  
the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Doctor of Philosophy

ISOLATION AND CHARACTERIZATION OF KDOP SYNTHASE AND TWO  
ISOZYMES OF DAHP SYNTHASE IN Spinacia oleracea L. AND  
Solanum tuberosum L.

by

RON LOU DOONG

December 1990

Chairman: Roy A. Jensen

Major Department: Microbiology and Cell Science

Three enzymes, capable of condensing PEP and E4P to form DAHP, were separated by DEAE-cellulose chromatography and identified as DAHP synthase-Mn (DS-Mn), DAHP synthase-Co (DS-Co), and KDOP synthase, respectively.

The plastidial isozyme, DS-Mn, was highly specific for both substrates, i.e., PEP could not be replaced by pyruvate, and E4P could not be substituted by glyceraldehyde 3-phosphate or glycolaldehyde. It was a hysteretic enzyme, and a lag was observed in standard assay conditions. The enzyme required DTT for activity, and could be further stimulated by  $Mn^{++}$ . EDTA at 0.05 mM concentration completely inhibited its activity. The optimum pH was 8.0, and the optimum temperature was 49°C for its activity. Activity was strongly inhibited by 0.7 mM

arogenate at pH 7.0. Thioredoxin could stimulate its activity only in the presence of DTT, suggesting that this enzyme is tightly regulated by light as are many other chloroplast enzymes involved in photosynthetic carbon assimilation. Its rapid induction by mechanical wounding indicates that the enzyme may be involved in the defense mechanism triggered by this stress condition.

The cytosolic isozyme, DS-Co, on the other hand, used an array of substrates with carbon length ranging from 2 to 4, glycolaldehyde being the best substrate tested based on its high specificity constant ( $V_{max}/K_m$ ). It required divalent metals for activity. At equimolar concentration of 0.5 mM,  $Co^{++}$  was the best,  $Mn^{++}$  the second, and  $Mg^{++}$  the third. The pH optimum was 9.5 and temperature optimum for activity was 49°C. The regulation of this enzyme has not been established, and a new role in plant metabolism is being investigated.

Owing to its substrate ambiguity, KDOP synthase was identified for the first time in higher plants. It possessed weak activity as 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase. In the presence of phosphoenolpyruvate, which conferred dramatic thermostability, KDOP synthase had a catalytic temperature optimum of about 53°C. The pH optimum was 6.2, and divalent cations were neither stimulatory nor required for activity. The  $K_m$  values for arabinose-5-P and phosphoenolpyruvate were 0.27 mM and about 35  $\mu M$ , respectively. The kinetics of periodate oxidation of KDOP



formed by spinach KDOP synthase indicate that the same stereochemical configuration exists as with bacterial KDOP.

## CHAPTER I LITERATURE REVIEW AND RATIONALE

Since the initial investigation on the formation of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) in sweet potato roots by Minamikawa and Uritani (1967), the number of isozymes and regulation of DAHP synthase in algae and higher plants has been a subject of controversy. Only one form of the enzyme had been reported in mung bean shoots (Minamikawa, 1967), Euglena gracilis (Weber and Bock, 1968), cauliflower florets (Huisman and Kosuge, 1974) before three isozymes with different pH optima were demonstrated in the cotyledons of Pisum sativum by the use of ammonium sulfate gradient solubilization in 1976 (Rothe et al.), although the regulation of the three isozymes was not investigated. In 1980 Graziana and Boudet reported that the extraction of the enzyme in the shoots of Zea mays required the presence of thiol compounds such as dithiothreitol or  $\beta$ -mercaptoethanol. The activity of the enzyme was inhibited in vitro by tryptophan. The enzyme was retarded on a tryptophan agarose affinity column, and it could be eluted with buffer containing tryptophan. Inhibition of the enzyme was pH dependent and the magnitude of inhibition increased during development. The sensitivity of the enzyme to tryptophan was rapidly lost even when stored at low

temperature. The specific activity of the enzyme from roots and shoots decreased with the development of corn seedlings. The enzyme from extracts of roots was found to be insensitive to tryptophan at any stage of the development. Isozymes could not be demonstrated in this monocot, yet this was the first report of a regulated DAHP synthase in higher plants. Inhibition of the enzyme by tyrosine was first reported by Byng et al. (1981) in Euglena gracilis, inhibition being noncompetitive with respect to either phosphoenolpyruvate or erythrose 4-phosphate. Reinink and Borstlap (1982) reported that the enzyme activity in the crude extracts from pea leaves was strongly inhibited by tyrosine and slightly stimulated by phenylalanine and tryptophan; the inhibition could also be relieved by the latter two aromatic amino acids. They concluded that about 85% of the enzyme activity was inhibitable and the remaining insensitive activity might be due to partial desensitization of the enzyme during the ammonium sulfate fractionation. In contrast, the enzyme in extracts from Daucus carota suspension-cultured cells was reported to be activated by tyrosine and tryptophan, the activation being dependent upon the time after transfer of the cells to fresh media (Suzich et al., 1984). The number of the isozymes from carrot roots was not studied (Suzich et al., 1984) until 1985 when three activities were separated by chromatography on phosphocellulose (Suzich et al., 1985). Enzyme III was hysteretic and could be activated by

physiological concentrations of tryptophan. The three isozymes also shared antigenic determinants.

Two differentially regulated isozymes of DAHP synthase with very different pH optima and regulatory properties, designated DS-Mn and DS-Co, were separated from seedlings of Vigna radiata [L.] Wilczek by DEAE-cellulose column chromatography (Rubin and Jensen, 1985). The activity of DS-Mn was activated by chorismate, and was inhibited by prephenate, L-arogenate, and tryptophan while DS-Co was not sensitive to allosteric control. The inhibition of DS-Mn by tryptophan was shown to increase with decreasing pH. This two-isozyme system was further demonstrated in extracts from suspension-cultured cells of Nicotiana glauca and leaves of tobacco (Ganson et al., 1986). DS-Mn required dithiothreitol for activity and was stimulated by manganese, while DS-Co required divalent metals for activity. Due to their diametrically different properties in the requirements of DTT and divalent metals for activation, pH optima, and substrate saturation concentrations with respect to erythrose-4-phosphate, selective assays are possible for the detection of either isozyme in a mixture of the two. DS-Mn was demonstrated to be localized in the chloroplast; whereas DS-Co was thought to be in the cytosol for there was little, if any, activity in the chloroplast. The two isozymes were shown to respond differently to transfer of the suspension-cultured cell of N. glauca to fresh media; DS-Mn activity declined substantially in stationary phase and

reached its peak in early exponential phase; whereas DS-Co activity peaked in late exponential phase. In contrast, only one enzyme, similar to DS-Mn, was reported in the tubers of Solanum tuberosum L. (Pinto et al., 1986) and a cDNA encoding this enzyme has been cloned (Dyer et al., 1990). Glyphosate was shown to induce the activity of this DAHP synthase in suspension-cultured cells of potato (Pinto et al., 1988) although it had no effect on the enzyme activity in vitro. A glyphosate-tolerant tobacco cell line, N. tabacum L. Indiana (17), was later selected from the glyphosate-sensitive line and demonstrated to have elevated DAHP synthase activity (Dyer et al., 1988).

Since potato and tobacco belong to the family of Solanaceae, it seems surprising that the biochemistry of these two plants would differ so much as to have one isozyme on the one hand and two isozymes on the other. Although DAHP synthases in spinach (Spinacia oleracea L.) have been investigated with crude extracts and extracts of purified chloroplast to establish their subcellular location, chromatographic separation has not yet been achieved. The objectives of this investigation are to establish the number of isozymes of DAHP synthase by DEAE-cellulose column chromatography in S. tuberosum and S. oleracea as they represent nonphotosynthetic and photosynthetic tissues, respectively in higher plants, and to further characterize the enzymes obtained from chromatography with respect to their

substrate specificities, pH and temperature optima, metal and thiol compound requirements, and regulation.



## CHAPTER II

### DAHP SYNTHASE-Mn (DS-Mn)

#### Introduction

DAHP synthase-Mn (DS-Mn) is the plastidial enzyme in higher plants for the committed step of aromatic amino acid biosynthesis. It has been partially purified and characterized in mung bean (Rubin and Jensen, 1985), tobacco suspension-cultured cells (Ganson et al., 1986) by DEAE-cellulose chromatography, and from spinach by chloroplast purification (Ganson et al., 1986). This enzyme is undoubtedly the one that catalyzes the condensation of PEP and E4P to form DAHP as the precursor for the synthesis of phenylalanine, tyrosine, and tryptophan in the chloroplast. The DAHP synthases documented in literature could be either DS-Mn or DS-Co on the basis of substrate ambiguity that was observed with DS-Co, requirement of reducing agents, or divalent cations for catalysis. Hence, the first DAHP synthase reported in mung bean shoots by Minamikawa (1967) seemed to be DS-Mn since the enzyme showed activity without divalent cations. The purified enzyme from cauliflower florets (Huisman and Kosuge, 1974) also behaved like DS-Mn for glyceraldehyde-3-phosphate or glyceraldehyde was not used as substrates. The DAHP synthase in corn (Graziana and Boudet, 1980) was probably the first DS-Mn type enzyme ever reported in monocots for its requirement of thiol

compounds in the extraction of the active enzyme, and for its activity without divalent cations. However, all of these investigations were made with plant tissues that are not photosynthetically active. The first report of DAHP synthase in higher plant tissues with vigorous photosynthetic activity was from pea leaves (Reinink and Borstlap, 1982); however, the enzyme was only partly purified by ammonium sulfate fractionation. DS-Mn in spinach leaves was detected in isolated chloroplast, yet simultaneous demonstration of the two isozymes by an ion-exchanger chromatography was never attempted. This chapter describes the response of the two isozymes to mechanical wounding, a fast and yet complete separation of DS-Mn from DS-Co and KDOP synthase in potato tubers and spinach leaves, its enzymological properties, and its regulation by intermediary metabolites of aromatic amino acid biosynthesis.

## Materials and Methods

### Plant Material

Spinach and Idaho potatoes were purchased from a local supermarket, washed with deionized water, frozen with liquid nitrogen, and ground to a fine powder by use of a Waring blender. The powders were stored at  $-70^{\circ}\text{C}$  prior to extract preparation.



### Plant Extract Preparation

All procedures were carried out at 0-4°C. A 45-g amount of powder was mixed with 30 ml of buffer A (50 mM K phosphate, pH 7.2, containing 0.5%  $\beta$ -mercaptoethanol) and thawed at room temperature. The extract was clarified by centrifugation at 29,000 x g for 30 min and filtered through Miracloth. A one-tenth volume of 2% protamine sulfate in buffer A was slowly added to the extract and stirred for 10 min. The precipitate was removed by centrifugation at 29,000 x g for 20 min.

Spinach and potato extracts used for column chromatography were further treated as follows. The foregoing supernatants were brought to 70% (spinach) or 60% (potato) of saturation with finely ground ammonium sulfate and stirred for 10 min. The protein precipitate was collected by centrifugation at 29,000 x g for 20 min, and resuspended in a minimal volume of buffer B (10 mM EPPS, pH 7.5, and 50 mM KCl). Desalting was accomplished by passage through Sephadex G-25 (PD-10) columns equilibrated with buffer B according to the manufacturer's instructions.

### DE-52 Column Chromatography of Plant Extracts and Enzyme Assays

A 150-mg (84-mg for potato) amount of protein was loaded onto a DE-52 anion exchanger column (1.5 x 19 cm) equilibrated with buffer B. The column was washed with 3 bed volumes of the buffer before a 400 ml gradient (50-300 mM KCl) in buffer B was applied. For the potato extracts a 400 ml gradient (50-500

mM KCl) was employed. The flow rate was 30 ml/hr, and fractions of 2.9 ml were collected.

### Enzyme Assays

Assays were executed at 37°C for 20 min unless otherwise indicated in 200  $\mu$ l reaction mixtures containing 50 mM EPPS (pH 8.0), 12.5 mM KCl, 3 mM PEP, 0.6 mM E4P, 0.5 mM DTT and 0.5 mM MnCl<sub>2</sub> for DS-Mn; 50 mM EPPS (pH 8.6), 40 mM KCl, 3 mM PEP, 3 mM E4P, and 10 mM MgCl<sub>2</sub> for DS-Co; 50 mM BTP (pH 6.5), 3 mM PEP, and 3 mM arabinose-5-P for KDOP synthase. The reaction was stopped with 50  $\mu$ l TCA (20%) and the enzymatic products were assayed as follows.

### Chemical Assays

DAHP was assayed as described by Jensen and Nester (1966), using the chemical method of Weissbach and Hurwitz (1959) as adapted by Srinivasan and Sprinson (1959). The absorbance at 549 nm was measured in a thermostatically controlled auto-sampler cuvette set at 55°C.

### Protein Assays

Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

### Mechanical Wounding of Potatoes

Tubers of Idaho potatoes were purchased from a local supermarket, washed with deionized water, and soaked in a streptomycin sulfate solution (25  $\mu$ g/ml). Disks of 12 mm diameter by 13 mm thickness were excised from the center tissue of the tubers and placed in petri dishes (15 cm

diameter) containing Whatman #3 filter paper saturated with streptomycin sulfate solution. The petri dishes were wrapped in aluminum foil and stored in a cabinet at room temperature. Disks harvested at zero time, 12, 24, 35, 48, 72, 96, 120, and 144 h, were frozen in liquid nitrogen and ground to a fine powder in a Waring blender. Powders were stored at  $-80^{\circ}\text{C}$  before use.

### Biochemicals

PEP (monocyclohexylammonium salt), glycolaldehyde, D-glyceraldehyde, L-glyceraldehyde, DL-glyceraldehyde-3-phosphate, D-erythrose, L-erythrose, D-threose, L-threose, D-erythrose-4-phosphate, protamine sulfate, and buffers (EPPS, BTP) were obtained from Sigma. DTT was purchased from Research Organics, (Cleveland, OH). Thioredoxin of *E. coli* was from CalBiochem, (La Jolla, CA). DE-52 anion exchanger was obtained from Whatman, Inc. (Clifton, NJ). PD-10 columns were from Pharmacia (Piscataway, NJ).

### Results

#### Construction of Standard Curve of DAHP Using Authentic KDO as a Standard

The extinction coefficient was calculated to be  $85,500 \text{ M}^{-1}\text{cm}^{-1}$  using authentic KDO as a standard, and this value was used throughout this dissertation.

#### Separation of DS-Mn from DS-Co by DE-52 Chromatography

Since the lability of DS-Mn from tobacco suspension-cultured cells was well appreciated, an attempt was made to

achieve a fast and complete separation of two isozymes of DAHP synthase with DEAE-cellulose chromatography. A buffer containing 10 mM EPPS at pH 7.5 and 50 mM KCl proved to serve this purpose. Under this condition DS-Mn eluted in the wash while DS-Co was retarded by the anion exchanger. A third enzyme, that proved to be KDOP synthase (see chapter IV), was also identified by virtue of its ability to utilize E4P as a cosubstrate (Fig. 2-1 and 2-2).

#### Thermostability

The enzyme was very labile even at room temperature in the absence of PEP. It lost about 10% of the activity as compared with the enzyme in the presence of PEP at 24°C for 30 min. At 37°C only 46% activity could be recovered when PEP was present; whereas 32% activity remained when PEP was absent during thermal treatment (Table 2-1).

#### pH and Temperature Optima

The reaction velocity of DS-Mn from spinach rose with pH up to 8.0, then dropped sharply with the pH higher than 8.0 (Fig 2-3). This was also true for DS-Mn from potato tuber. Temperature optimum was 49°C, similar to that of DS-Co (Fig. 2-4).

Fig. 2-1. Elution profiles of potato DS-Mn, DS-Co, and KDOP synthase from DEAE cellulose. An 84-mg amount of potato extract prepared as described in Materials and Methods was applied. The salt gradient was 50 to 500 mM KCl in buffer B, also containing 0.5 mM PEP. The  $A_{549}$  values plotted on the ordinate were obtained by incubation of 100  $\mu$ l aliquots of enzyme for 40 min (KDOP synthase), 30 min (DS-Mn), and 20 min (DS-Co).

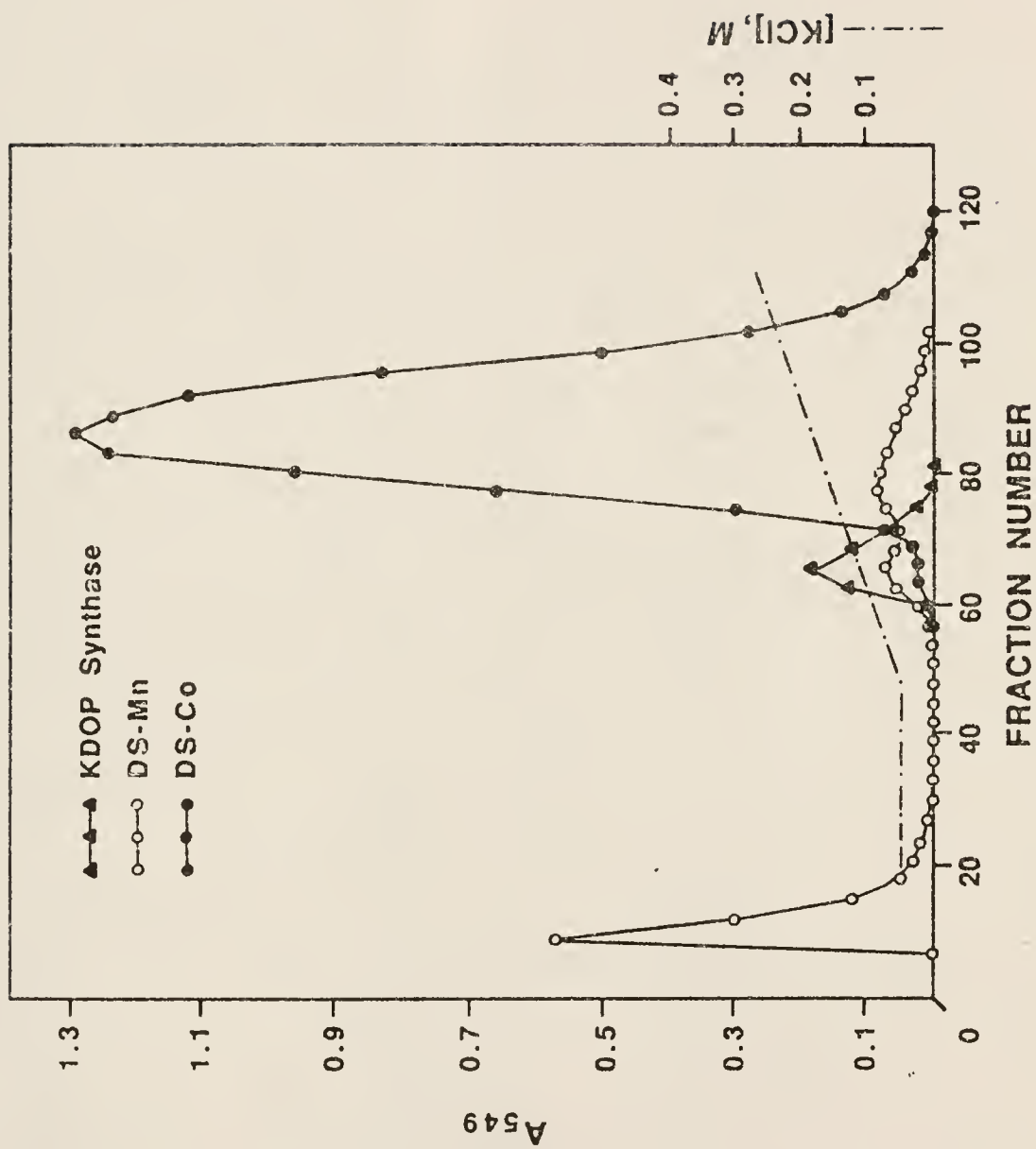


Fig. 2-2. Elution profiles of spinach DS-Mn, DS-Co, and KDOP synthase from DEAE cellulose. A 150-mg amount of spinach extract prepared as described in Materials and Methods was applied. The salt gradient was 50 to 300 mM KCl in buffer B. The  $A_{549}$  values plotted on the ordinate scale were obtained by incubation of 50  $\mu$ l aliquots of enzyme for 20 min at 37°C.

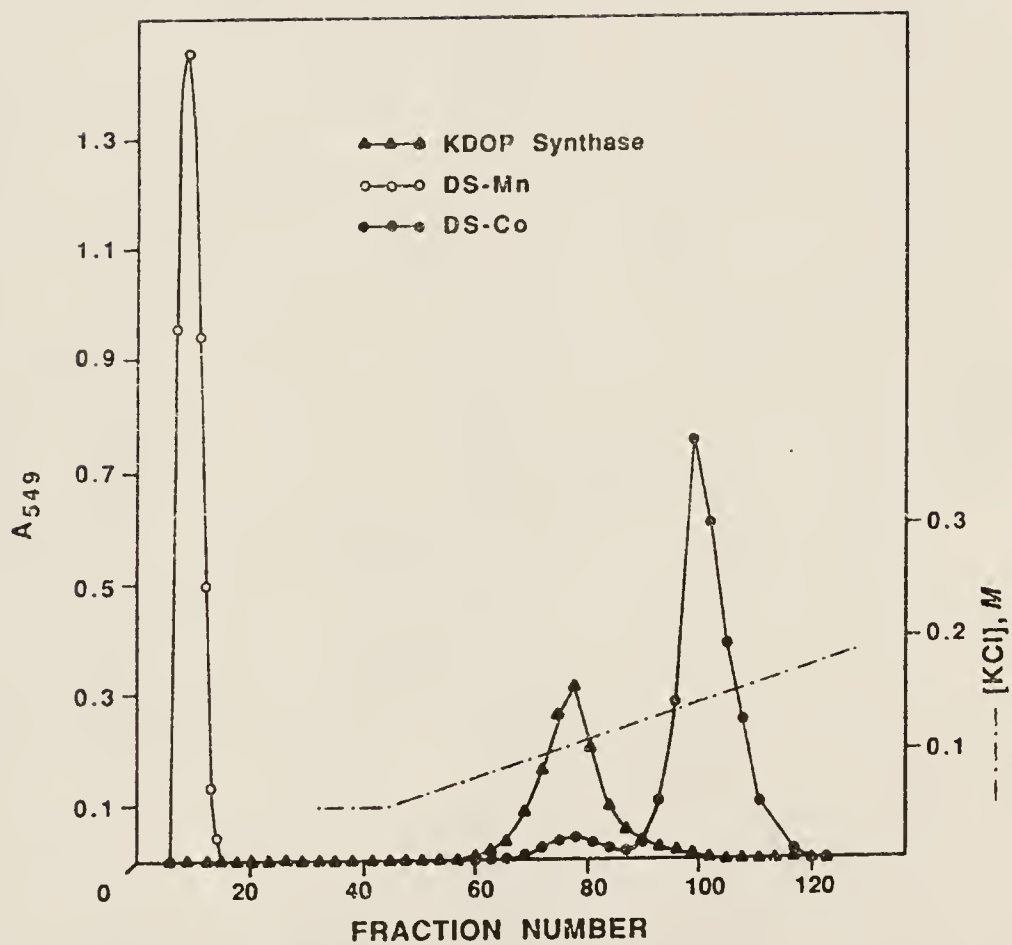




TABLE 2-1. Thermostability of spinach DS-Mn

<u>Thermal treatment</u>	<u>% of control activity</u>	
	<u>+ PEP</u>	<u>- PEP</u>
24°C	100	89
37°C	46	32
47°C	7	5
57°C	0	0

Partially purified enzyme was incubated with or without 1.1 mM PEP at the indicated temperature for 30 min and removed to an ice bath prior to assay at 37°C under standard conditions. 100% activity is equivalent of 19.8 nmol.

#### Substrate Specificity

DS-Mn exhibited an absolute specificity for both PEP and E4P. Thus, PEP could not be replaced by pyruvate, and E4P could not be replaced by any of the alternative sugar substrates such as glycolaldehyde, D-glyceraldehyde, L-glyceraldehyde, DL-glyceraldehyde-3-phosphate, D-erythrose, L-erythrose, D-threose, or L-threose.

#### Divalent Metal Effects

Unlike DS-Co, DS-Mn from spinach did not require divalent cations for activity; however, at optimal concentration of 0.5 mM, its activity could be further enhanced 47% and 9% by  $\text{MnCl}_2$  and  $\text{MgCl}_2$ , respectively (Fig. 2-5). At the same concentration of  $\text{CoCl}_2$ , inhibition of 60% was observed. EDTA at the concentration of 0.05 mM completely inhibited the activity.

Dialysis of the enzyme treated with 1 mM EDTA against buffer containing DTT only restored 15% of the activity when assayed without divalent metal; however, upon the addition of 0.5 mM  $\text{MnCl}_2$  or  $\text{CaCl}_2$ , the activity was restored 90% and 52%, respectively, although  $\text{CaCl}_2$  did not stimulate the non-EDTA treated enzyme activity. This indicated that the enzyme might be a metalloprotein whose divalent cation could be readily chelated by EDTA. This plastidial enzyme differs from DS-Co, the cytosolic isozyme, in that the latter requires divalent metals for activity.

Fig. 2-3. pH optima of DS-Mn and DS-Co from spinach. Reaction mixtures contained 3 mM PEP, 0.6 mM E4P, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM BTP buffer at indicated pH for DS-Mn; 3 mM PEP, 3 mM E4P, 10 mM  $\text{MgCl}_2$ , 40 mM KCl, 50 mM BTP or CHES buffer at indicated pH for DS-Co. Incubation was 10 min for DS-Mn, and 20 min for DS-Co at 37°C.

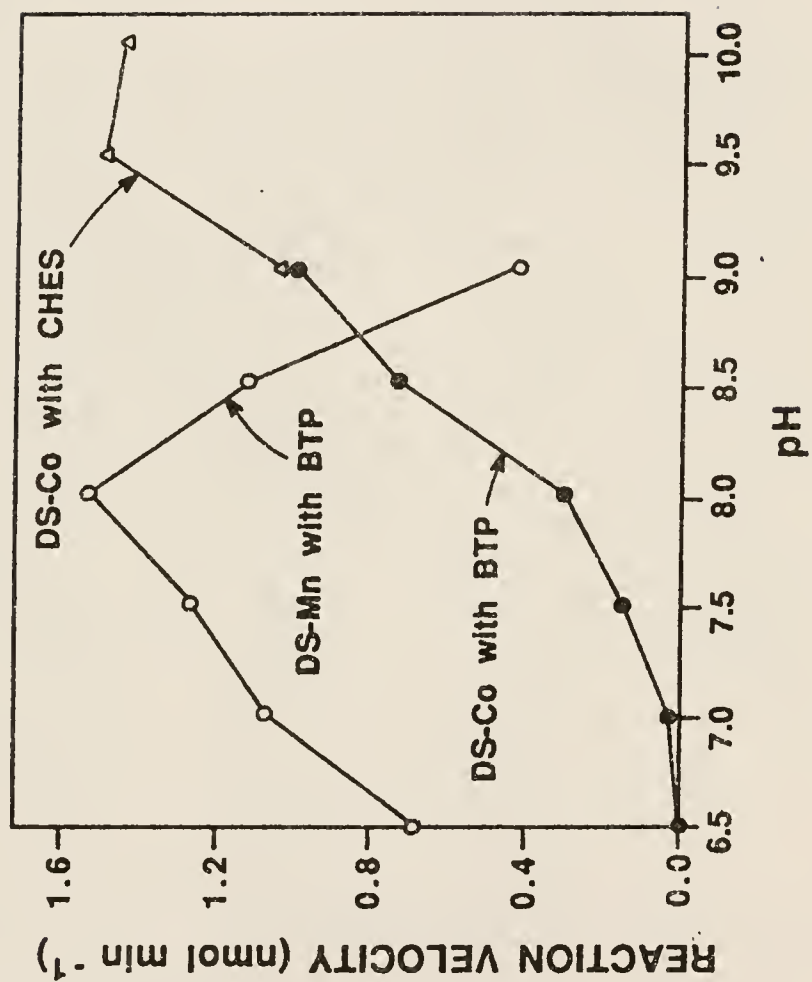
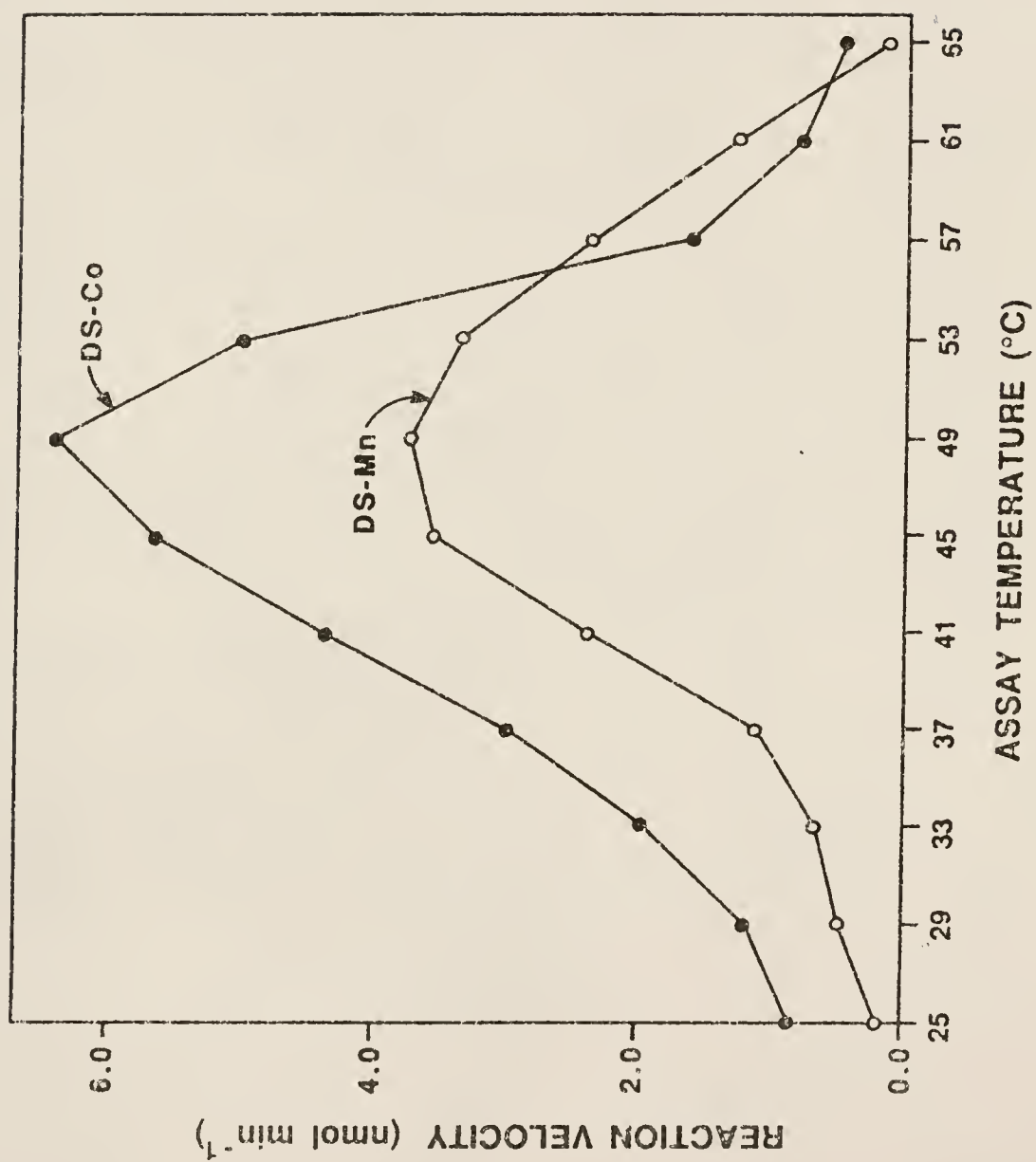


Fig. 2-4. Temperature optima of DS-Mn and DS-Co from spinach. Reaction mixtures contained 3 mM PEP, 0.6 mM E4P, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM EPPS buffer at pH 8.0 for DS-Mn; 3 mM PEP, 3 mM E4P, 10 mM  $\text{MgCl}_2$ , 75 mM KCl, 50 mM EPPS at pH 8.6 for DS-Co. Incubation was 5 min at indicated temperature for both.



### Effects of Dithiothreitol and Thioredoxin

The enzyme required dithiothreitol for activity. In the presence of DTT, activity was increased two-fold by 25  $\mu$ g of bacterial thioredoxin with partially purified enzyme. In the absence of DTT, thioredoxin did not have any effect on the enzyme (Fig. 2-6). The hysteretic lag of the progress curve could be removed by pre-assay incubation of the enzyme with PEP and DTT (Fig. 2-7). This is similar to many enzymes in chloroplasts that are regulated by light. These redox properties of DS-Mn were not observed for the cytosolic isozyme, DS-Co. The stimulation of the enzyme in the crude extracts of spinach by thioredoxin was not so remarkable as that observed with crude extracts of potato (Table 2-2). Perhaps the spinach extracts are relatively rich in endogenous reduced thioredoxin compared to nonphotosynthetic potato tubers.

### Substrate Saturation Curves

Spinach DS-Mn showed sigmoid substrate saturation curves with both E4P and PEP. E4P exhibited substrate inhibition at concentrations higher than 0.6 mM. The enzyme was saturated by 3 mM PEP (Fig. 2-8 and 2-9).

Fig. 2-5. Divalent metal effects on spinach DS-Mn. Reaction mixtures contained 3 mM PEP, 0.6 mM E4P, 0.5 mM DTT, 12.5 mM KCl, 50 mM EPPS at pH 8.0, and  $\text{MnCl}_2$  or  $\text{MgCl}_2$  at indicated concentrations. Incubation was 20 min at 37°C.



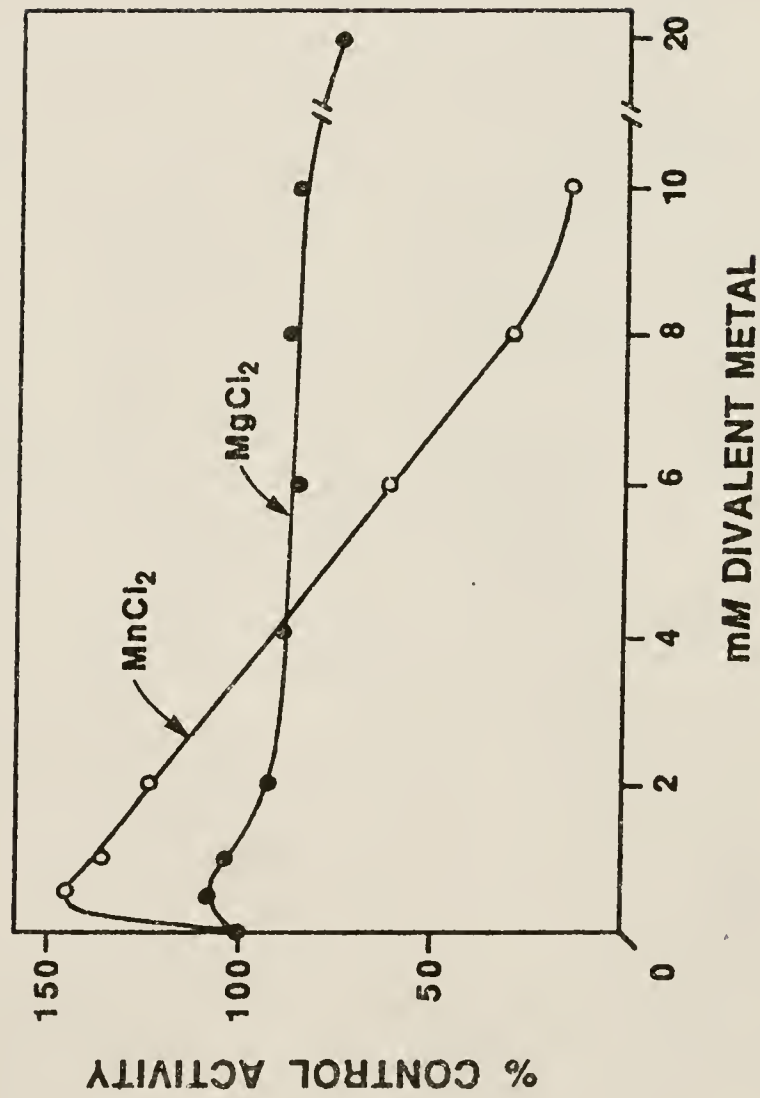


Fig. 2-6. Effects of DTT and thioredoxin on spinach DS-Mn. Reaction mixtures contained 3 mM PEP, 0.6 mM E4P, 0.5 mM  $\text{MnCl}_2$ , 12.5 mM KCl, 50 mM EPPS at pH 8.0. DTT and thioredoxin was 0.5 mM and 12.5  $\mu\text{g}$ , respectively, if present. The reaction was followed for 30 min. The progress curve with thioredoxin in the absence of DTT superimposes with the curve obtained without either.

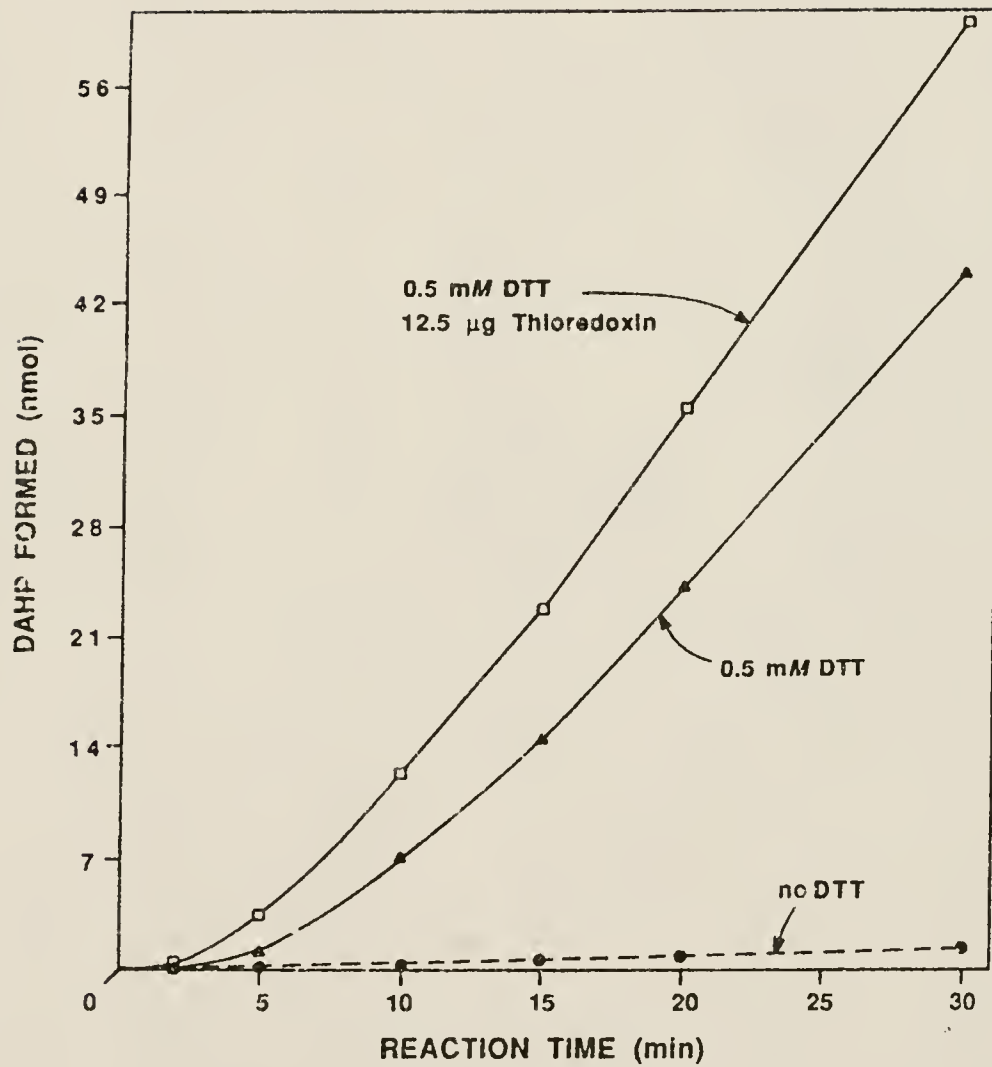


Fig. 2-7. Progress curves of fully activated spinach DS-Mn. Reaction mixtures containing 3 mM PEP, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM EPPS at pH 8.0, and enzyme were incubated at 37°C for 15 min before E4P was added at a final concentration of 0.6 mM. The reaction was followed for 30 min.

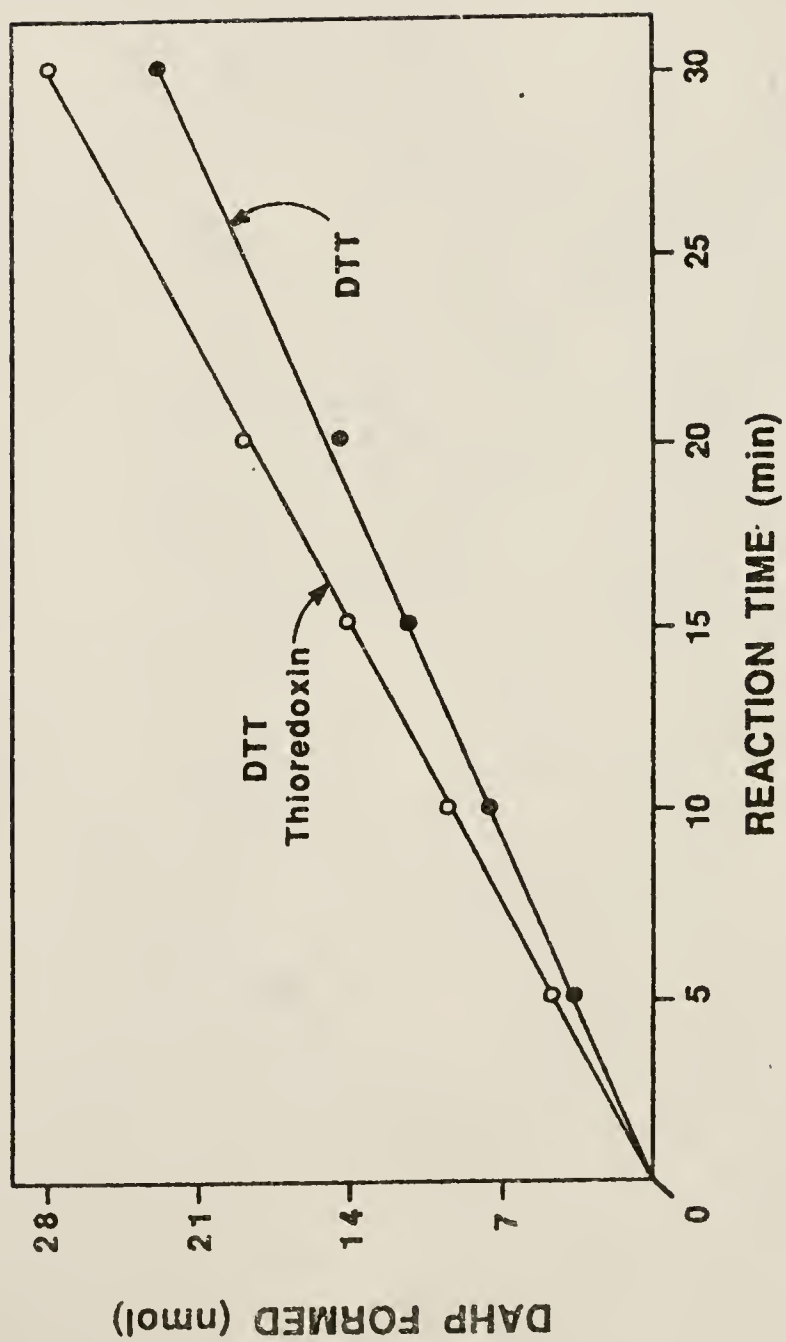


TABLE 2-2. Effect of DTT and/or thioredoxin on DS-Mn activity from crude extracts of potato tubers and spinach leaves.

<u>Assay conditions</u>	<u>A<sub>549</sub></u>	
	<u>Potato</u>	<u>Spinach</u>
No Addition	0.058	0.328
DTT (0.5 mM)	1.38	0.681
Thioredoxin (12.5 $\mu$ g)	0.081	0.349
DTT + Thioredoxin	1.864	0.745

Extracts were made with 50 mM K-Phosphate buffer at pH 7.2 and 0.1%  $\beta$ -mercaptoethanol. Clarified extracts were desalted into 10 mM EPPS buffer pH 8.0 and 50 mM KCl by passage through PD-10 columns.

#### Effect of $\beta$ -Mercaptoethanol on the Extraction of DS-Mn

Similar to the enzyme in tobacco suspension-cultured cells, DS-Mn in potato tubers could only be detected when  $\beta$ -mercaptoethanol was present in the extraction buffer. It differed from DS-Mn in spinach leaves where the activity of DS-Mn could be readily measured in the crude extracts prepared without  $\beta$ -mercaptoethanol (Table 2-3). This might be due to the presence of reduced thioredoxin in spinach leaf extracts and its absence in potato tubers. Potatoes are rich in phenolic compounds that may contribute to inactivate the enzyme in the absence of reducing agents.

TABLE 2-3. Effect of  $\beta$ -mercaptoethanol on the extraction of DS-Mn in potato tubers and spinach leaves

<u>Assay Conditions</u>	<u>Extraction Condition</u>			
	<u>No <math>\beta</math>ME</u>		<u>0.5% <math>\beta</math>ME</u>	
	<u>potato</u>	<u>spinach</u>	<u>potato</u>	<u>spinach</u>
No thiol reagent	0.027	0.358	0.186	1.053
DTT (mM) 0.125	0.021	0.793	0.846	1.209
0.25	0.017	0.875	1.077	1.145
0.5	0.013	0.903	1.30	1.055
1.0	0.009	0.852	1.235	0.904

Crude extracts were made with 50 mM KP buffer, pH 7.2, with or without 0.5%  $\beta$ -mercaptoethanol. The clarified extracts were desalted into 10 mM EPPS buffer, pH 8.0, 50 mM KCl with PD-10 columns.  $A_{549}$  was recorded after a 20 min duration of reaction.

Effects of Aromatic Amino Acids and Their Intermediate Metabolites on Spinach DS-Mn at Various pH's

DS-Mn was found to be sensitive to arogenate. The inhibition was a function of pH; at pH 7.0, 65% of the activity was inhibited, while at pH 8.0 only 23% of the activity was inhibited. The concentration for 50% inhibition was estimated to be 0.5 mM at pH 7.0 (Fig. 2-10). The inhibition by arogenate was competitive and noncompetitive with respect to E4P and PEP, respectively. A slight activation of 10% was observed when the pH was raised to 9.0. Phenylalanine, tyrosine, tryptophan, chorismate, prephenate,

and caffeic acid did not have much effect on the enzyme (Table 2-4).

TABLE 2-4. Effects of aromatic amino acids and intermediary metabolites on spinach DS-Mn at different pH's

<u>effectors</u>	<u>% control activity</u>		
	<u>pH 7.0</u>	<u>pH 8.0</u>	<u>pH 9.0</u>
PHE	109	99	91
TYR	110	94	102
TRP	97	98	100
CHA	95	108	110
PPA	110	105	100
AGN	35	77	110

AGN was used at 0.7 mM, all others were 0.5 mM. Reaction tubes where respective effectors were added after enzyme reaction was stopped with TCA served as controls. PHE, TYR, TRP, CHA, PPA, and AGN stand for phenylalanine, tyrosine, tryptophan, chorismate, prephenate, and arogenate, respectively.

#### Induction of DS-Mn after Mechanical Wounding of Potato Tubers

DS-Mn responded to mechanical wounding more rapidly and in greater magnitude than DS-Co. Within 48 hours after wounding treatment, the specific activity of DS-Mn increased about seven-fold, while DS-Co responded slowly with only a 15% increase in the same period (Fig. 2-11). Mechanical



wounding provides a means of enriching DS-Mn in vivo for the purpose of enzyme purification.

### Selective Assays

The finding that DS-Co utilizes glycolaldehyde and G3P facilitates a selective assay for DS-Co in the presence of DS-Mn. Hence, when assayed with E4P under selective assay conditions designed for DS-Co, DS-Mn showed 14% of its activities in the disguise of DS-Co; when assayed with glycolaldehyde or G3P as cosubstrates for DS-Co, DS-Mn activity was completely discriminated. This provides a convenient method to confirm if DS-Mn preparation is contaminated with DS-Co in the process of purification.

Fig. 2-8. Saturation curve of spinach DS-Mn by PEP. Reaction mixtures contained 0.6 mM E4P, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM EPPS at pH 8.0, and PEP at the indicated concentrations. Velocity was calculated from the linear section of the progress curve.

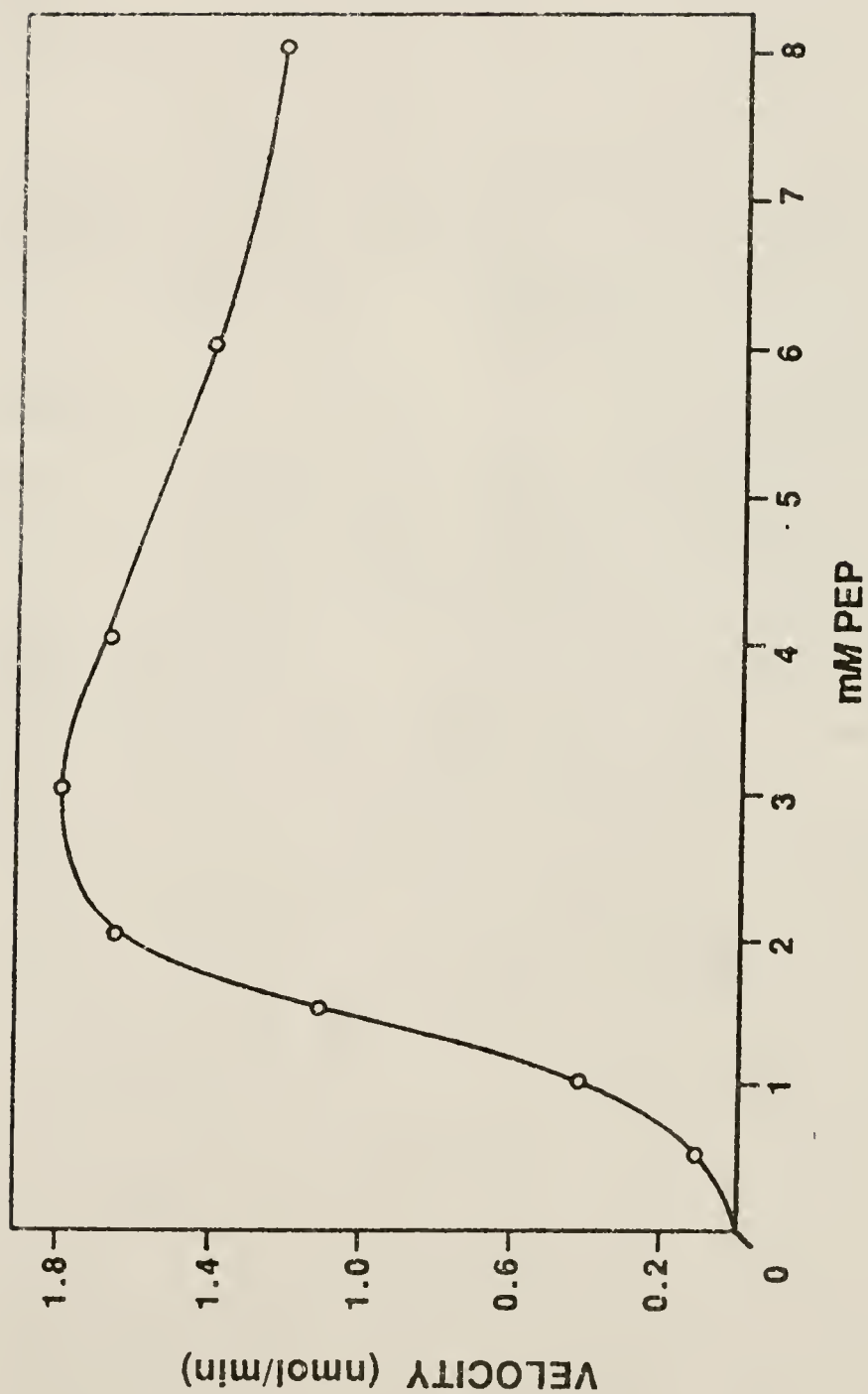


Fig. 2-9. Saturation curve of spinach DS-Mn by E4P. Reaction mixtures contained 3 mM PEP, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM EPPS at pH 8.0, and E4P at the indicated concentrations. Velocity was calculated from the linear section of the progress curve.

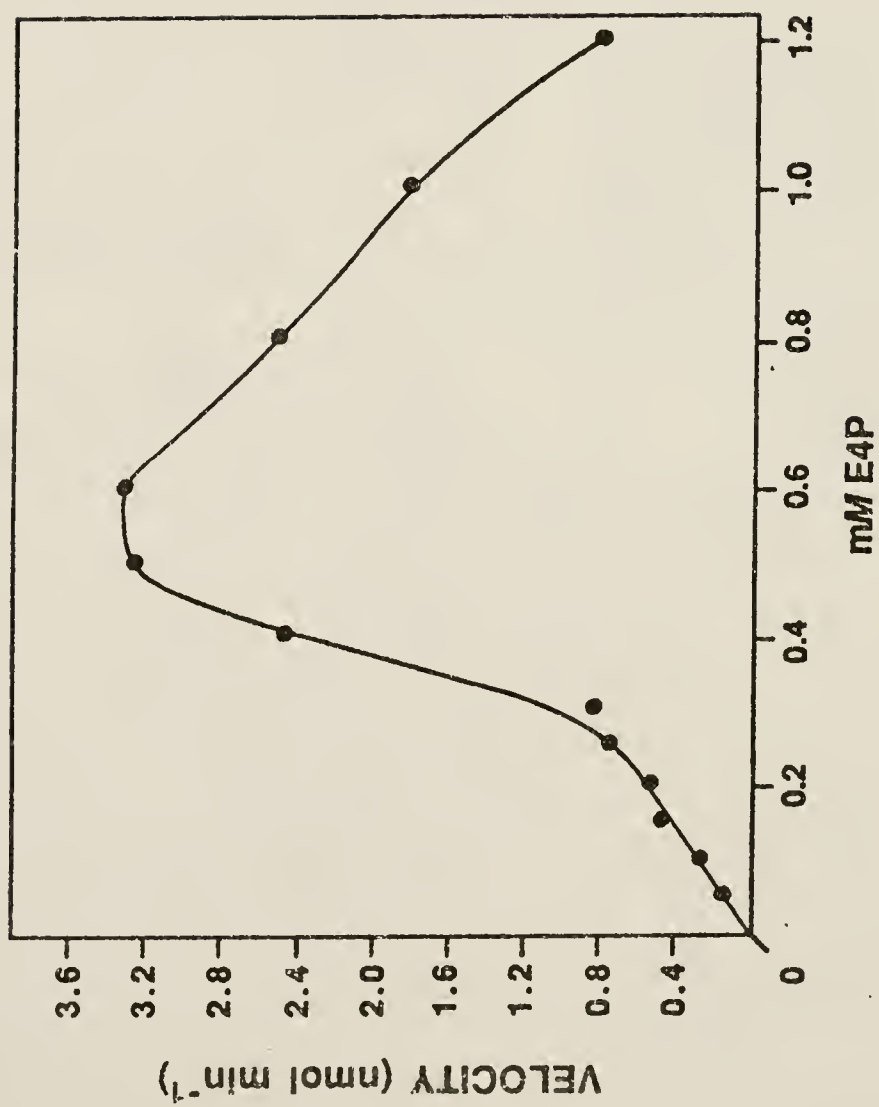


Fig. 2-10. Inhibition curve of spinach DS-Mn by arogenate. Reaction mixtures contained 3 mM PEP, 0.5 mM E4P, 0.5 mM  $\text{MnCl}_2$ , 0.5 mM DTT, 12.5 mM KCl, 50 mM BTP at pH 7.0 and arogenate at indicated concentrations. Incubation was 20 min at 37°C.

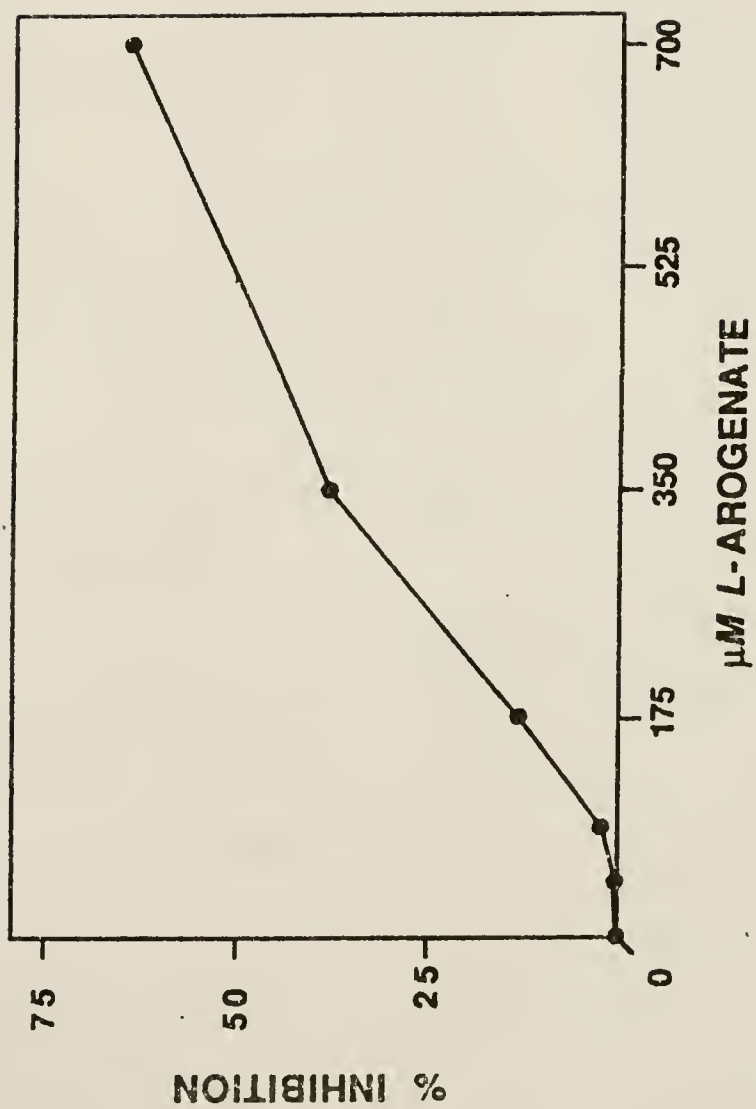
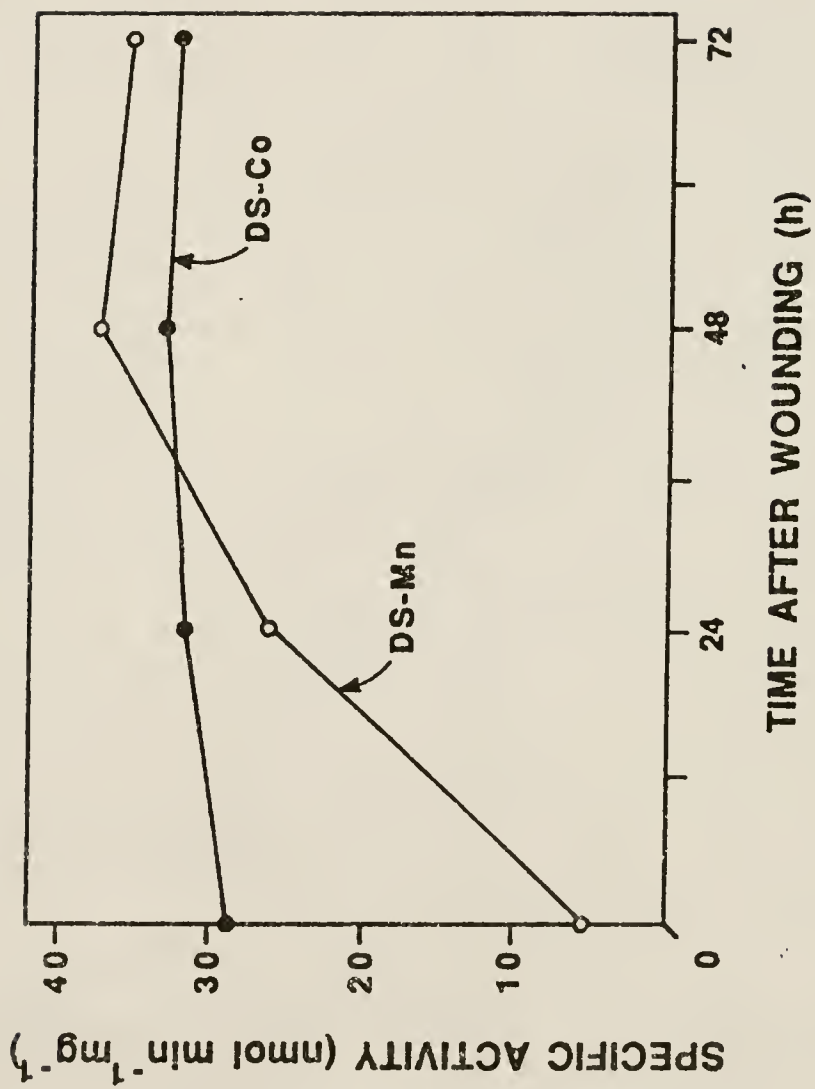


Fig. 2-11. Induction of DS-Mn and DS-Co in potato tubers by mechanical wounding.  
The crude extracts were made as described previously (Morris et al., 1989).





### Discussion

DS-Mn is responsible for the first enzymatic step in the biosynthesis of aromatic amino acids in the chloroplast. To resolve the confusion over the number of isozymes in higher plants in general and potato tubers in particular, results of mechanical wounding on potato tubers lend a strong support to the occurrence of a second isozyme, i.e., DS-Co. Complete chromatographic separation of two activities by DEAE cellulose further provided solid evidence for dual pathway hypothesis. DS-Mn has been shown to elevate rapidly in suspension-cultured cells of N. silvestris in response to subculture (Ganson and Jensen, 1987). Wounding might trigger what is in essence a growth response (Morris et al., 1988). In fact, wounding was shown to induce mRNA encoding DAHP synthase followed by the increased synthesis of the enzyme in potato or tomato tissue, and a similar effect was also observed with phenylalanine ammonia-lyase (Dyer et al., 1989). Induction of DS-Mn, not DS-Co, in cultured parsley cells by a cell wall fraction of the fungus Phytophthora megasperma was also reported (McCue and Conn, 1989). The nonresponsiveness of the enzyme to mechanical wounding and its activity without DTT reported in sweet potato roots by Minamikawa and Uritani (1966) indicated that the enzyme activity detected was obviously not that of DS-Mn. It is intriguing that DS-Mn responded much faster than its cytosolic counterpart while most, if not all, enzymes of secondary metabolism function in the cytosol (Jensen, 1986).

There might be a rapid export of phenylalanine into the cytosol after the induced biosynthesis of this precursor for secondary metabolism.

The requirement of DTT for activation and further enhancement by bacterial thioredoxin suggested that DS-Mn, like many chloroplast enzymes involved in the photosynthetic carbon assimilation, is also tightly regulated by light (Buchanan, 1980; Crawford et al., 1986; Jacquot et al., 1984; Jensen, 1986). Two forms of thioredoxin have been reported in the chloroplast of spinach, i.e., thioredoxin f and thioredoxin m (Wolosiuk et al., 1979). The question of which one is capable of stimulating DS-Mn activity merits further investigation. Stimulation by thioredoxin of shikimate kinase from spinach chloroplast has also been reported (Schmidt and Schultz, 1987), but the effect was later shown to be stabilization rather than activation (Schmidt et al., 1990). Induction of phenylalanine ammonia-lyase by light in potato tuber disks (Zucker, 1968; Sacher et al., 1972) paralleled by the increase of chlorogenic acid (Zucker, 1965) have also been demonstrated. In view of the parallel response of DS-Mn and PAL to mechanical wounding, stimulation by reduced thioredoxin, induction by the light, these two enzymes seem to play a concerted role in the synthesis of secondary metabolites such as phenylpropanoids and phytoalexins as a defense mechanism.

Both inhibition and activation of DAHP synthase by aromatic amino acids have been reported in higher plants although in most cases insensitivity was observed. A pH dependent inhibition of the enzyme in corn was also reported with respect to tryptophan in corn (Grazinia and Boudet, 1980) and in seedlings of Vigna radiata (Rubin and Jensen, 1985). In both cases the magnitude of inhibition increased with decreasing pH. On the other hand, tryptophan was reported to activate the enzyme from carrot roots (Suzich et al., 1985) and potato tubers (Pinto et al., 1986). Tyrosine was shown to be inhibitory for the enzyme from pea leaves (Reinink and Borstlap, 1982), but stimulatory for the enzyme from suspension-culture cells of carrot (Suzich et al., 1984). In this investigation, DS-Mn from spinach leaves could be demonstrated to be inhibited only by arogenate, and the inhibition was pH dependent, similar to the inhibition of chorismate mutase I (the chloroplast isozyme in the aromatic amino acid biosynthetic pathway) by tyrosine and phenylalanine (Goers and Jensen, 1984). The greater inhibition at pH 7.0 than at pH 8.0 suggests that in the light the enzyme is more active than in the dark. This pattern of regulation is consistent with a sequential model of feedback inhibition of aromatic amino acid biosynthesis in the chloroplast (Gaines et al., 1982; Jensen, 1985; Jung et al., 1986).

### CHAPTER III DAHP SYNTHASE-Co (DS-Co)

#### Introduction

Since two isozymes of DAHP synthase were established in higher plants such as mung beans, tobacco, spinach, and potatoes, the in vivo function of the cytosolic form of the isozyme DS-Co has not been established. In this investigation, the enzyme from spinach leaves and potato tubers (representative of photosynthetic and nonphotosynthetic tissues, respectively) was completely separated from DS-Mn by DEAE-cellulose column chromatography and has been further characterized in comparison with its chloroplast counterpart. Substrate specificity of the enzyme was extensively investigated as a potential role of the enzyme in a new biochemical pathway was suspected.

#### Materials and Methods

##### Plant Material

Spinach and Idaho potatoes were purchased from a local supermarket. All plant material was washed with deionized water, frozen with liquid nitrogen, and ground to a fine powder by use of a Waring blender. The powders were stored at -70°C prior to extract preparation.

### Plant Extract Preparation

All procedures were carried out at 0-4°C. A 45 g amount of powder was mixed with 30 ml of buffer A (50 mM K phosphate, pH 7.2, containing 0.5%  $\beta$ -mercaptoethanol) and thawed at room temperature. The extract was clarified by centrifugation at 29,000 x g for 30 min and filtered through Miracloth. A one-tenth volume of 2% protamine sulfate in buffer A was slowly added to the extract and stirred for 10 min. The precipitate was removed by centrifugation at 29,000 x g for 20 min.

Spinach and potato extracts used for column chromatography were further treated as follows. The foregoing supernatants were brought to 70% (spinach) or to 60% (potato) of saturation with finely ground ammonium sulfate and stirred for 10 min. The protein precipitate was collected by centrifugation at 29,000 x g for 20 min and resuspended in a minimal volume of buffer B (10 mM EPPS, pH 7.5, and 50 mM KCl). Desalting was accomplished by passage through Sephadex G-25 (PD-10) columns equilibrated with buffer B according to the manufacturer's instructions.

### DE-52 Column Chromatography of Plant Extracts and Enzyme Assays

150 mg spinach protein (or 84 mg potato protein) was loaded onto a DEAE-cellulose column (1.5 x 19 cm) equilibrated with buffer B. The column was washed with 3 bed volumes of buffer before a 400 ml gradient (50-300 mM KCl) in buffer B was applied. For the potato extracts a 400 ml gradient (50-500 mM KCl) was employed. The flow rate was 30 ml/hr, and



fractions of 2.9 ml were collected. The enzyme assays were carried out as described previously in Chapter II unless otherwise stated.

### Chemical Assays

The analytical assays were carried out as described in Chapter II except that periodate oxidation was 10 min for the enzymatic products with glyceraldehyde 3-phosphate, 20 min for the products with glycolaldehyde, and 30 min for the products with other sugars.

### Biochemicals

PEP (monocyclohexylammonium salt), glycolaldehyde, glyoxylate, D-glyceraldehyde, L-glyceraldehyde, DL-glyceraldehyde 3-phosphate, D-erythrose, L-erythrose, D-threose, L-threose, D-erythrose-4-phosphate, D-ribose, L-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-lyxose, L-lyxose, D-ribose-5-phosphate, D-arabinose-5-phosphate, D-glucose-6-phosphate, protamine sulfate, and buffers (EPPS, BTP, and CHES) were obtained from Sigma. DTT was purchased from Research Organics, (Cleveland, OH), and DE-52 Anion exchanger was obtained from Whatman, Inc. (Clifton, NJ). PD-10 columns were from Pharmacia (Piscataway, NJ).

## Results

### Separation of DS-Co from DS-Mn and KDOP Synthase by DE-52 Anion Exchanger Chromatography

DS-Co from spinach leaves and potato tubers both eluted from the anion exchanger at about 0.15 M KCl (Fig. 2-1 and 2-

2). A minor peak preceding the peak activity of DS-Co was detected in both cases under the assay condition for DS-Co. Unlike DS-Mn and DS-Co, this new activity did not require DTT or metal, which were required for DS-Mn or DS-Co respectively, for activation (Morris et al., 1989). It was later confirmed to be a new enzyme, i.e., KDOP synthase (see Chapter IV of this dissertation).

#### Thermostability

The enzyme was unstable in the absence of PEP; it lost more than 90% of activity after incubation at 37°C for 30 min. However, in the presence of PEP, 90% of the activity remained (Table 3-1).

#### pH and Temperature Optima

The enzyme was almost inactive at neutral pH. Activity rose with the pH and reached its peak at pH around 9.5. This could further facilitate the fine-tuning of selective assay for DS-Co in the presence of DS-Mn. The enzyme had a temperature optimum at 49°C, but was rapidly inactivated at higher temperatures (Fig. 2-3 and 2-4).

#### Saturation Curves of the Enzyme by PEP with Various Cosubstrates

DS-Co was saturated by 1.5 mM PEP when assayed with 3 mM E4P, 6mM G3P, or 8 mM glycolaldehyde. All the curves were sigmoid.



TABLE 3-1. Thermostability of spinach DS-Co

<u>Thermal treatment</u>	<u>% of control activity</u>	
	<u>+ PEP</u>	<u>- PEP</u>
27°C	100	86
37°C	90	5
47°C	4	1
57°C	0	0

Partially purified enzyme was incubated with or without 1.1 mM PEP at the indicated temperature for 30 min and removed to an ice bath prior to assay at 37°C under standard conditions.

#### Divalent Metal Requirement

Like the enzyme from tobacco, DS-Co from spinach or potatoes exhibited no activity without divalent metal. At the equimolar concentration of 0.5 mM,  $\text{Co}^{++}$  was the best,  $\text{Mn}^{++}$  the second, and  $\text{Mg}^{++}$  the third, with relative velocity of 100, 16, and 10, respectively.  $\text{Mn}^{++}$  and  $\text{Co}^{++}$  became inhibitory as the concentrations went higher than 1 mM, while  $\text{Mg}^{++}$  showed its maximal activation at 20 mM. Hence, at their maximal activation concentration (20 mM for  $\text{Mg}^{++}$  and 1 mM for  $\text{Co}^{++}$  and  $\text{Mn}^{++}$ ), the relative velocity was 100, 70, and 15, respectively (Fig. 3-1).

### Substrate Ambiguity

DS-Co from spinach and potato had the same properties with respect to substrate specificity, i.e., they both utilized an array of sugars ranging from carbon length of 2 to 4 to make the corresponding 2-keto-3-deoxy sugar acids, as shown in Table 3-2. Glycolaldehyde exhibited the highest reaction velocity when all sugars were used at 3mM concentrations. Pentoses were poor substrates except when phosphorylated, an effect due to the stabilization by the phosphate group of a significantly greater percentage of the open-chain form of pentose phosphates such as D-ribose 5-phosphate and D-arabinose 5-phosphate. No discrimination between the stereoisomers was observed as comparable velocities were demonstrated with either D or L isomer of glyceraldehyde, erythrose, or threose. Surprisingly, glyoxylate was also usable in this enzymatic reaction. The enzyme was obviously not a reversible aldolase since pyruvate failed to substitute for PEP.

On the basis of Michaelis constant, D-erythrose 4-phosphate seemed to be the best substrate because it had a  $K_m$  value of 1.95 mM. However, glycolaldehyde had the largest  $V_{max}$  value and a largest  $V_{max}/K_m$  value although it had a very large  $K_m$  value compared with erythrose 4-phosphate (Table 3-3).

Fig. 3-1. Divalent metal effects on spinach DS-Co. Reaction mixtures contained 2mM PEP, 3mM E4P, 50 mM EPPS, pH 8.6, 40 mM KCl, and divalent cation concentrations as indicated. Incubation was at 37°C for 20 min. Maximal activities are 1.94 nmol/min, 1.37 nmol/min, and 0.29 nmol/min, for  $Mg^{++}$ ,  $Co^{++}$ , and  $Mn^{++}$ , respectively.

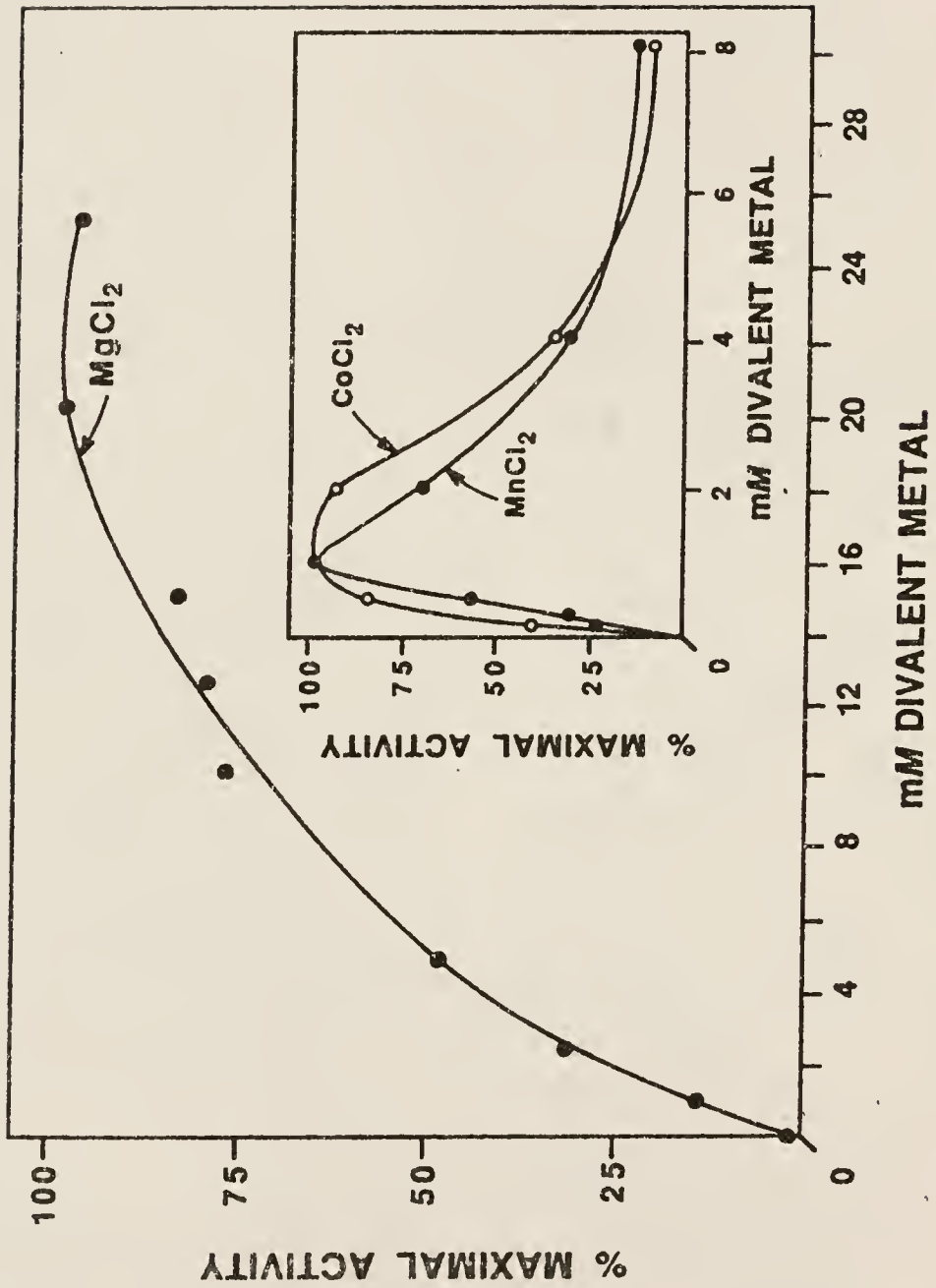


Fig. 3-2. Saturation curves of spinach DS-Co by PEP with various cosubstrates. The reaction mixture contained 10 mM  $\text{MgCl}_2$ , 40 mM KCl, 50 mM EPPS at pH 8.6, 3 mM E4P, (or 6 mM DL-G3P, or 8 mM glycolaldehyde), and PEP at indicated concentrations.

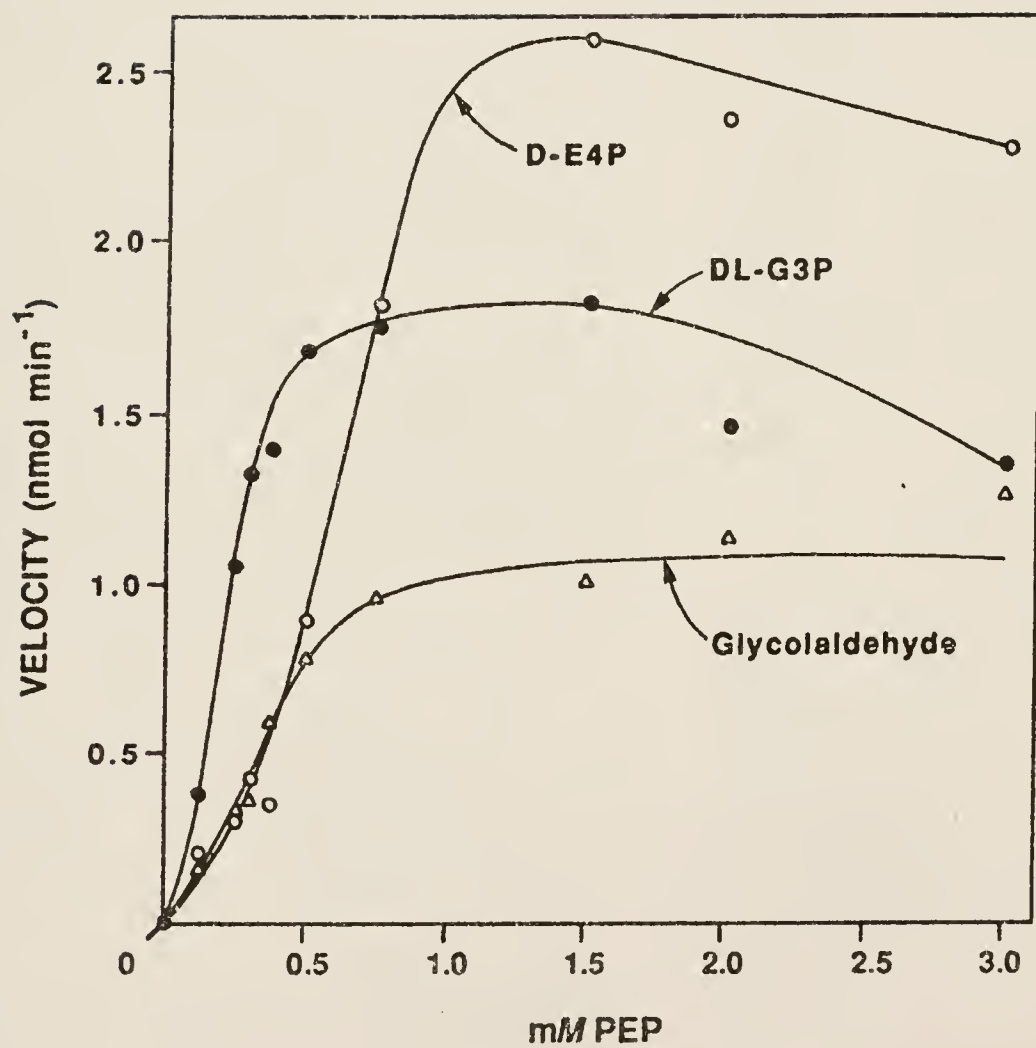


TABLE 3-2. Relative velocity of spinach DS-Co with various substrates

<u>Substrates</u>	<u>Relative Velocity</u>
glycolaldehyde	245
D-glyceraldehyde	176
L-glyceraldehyde	212
DL-glyceraldehyde-3-phosphate	142
D-erythrose	93
L-erythrose	70
D-threose	92
L-threose	52
D-erythrose-4-phosphate	100 (1.98 nmol/min)
D-ribose	1
L-ribose	1
D-arabinose	0.7
L-arabinose	0
D-xylose	1
L-xylose	1
D-lyxose	1.8
L-lyxose	1.8
D-ribose-5-phosphate	12
D-arabinose-5-phosphate	8
D-glucose-6-phosphate	0.5
glyoxylate	20

All the substrates above were present at 3 mM concentrations for standard assays.

### Confirmation of Reaction Products

The reaction products all had an absorption peak at 549 nm by periodate-thiobarbituric acid procedure, indicating the presence of 3-deoxy aldulosonic acid similar to DAHP molecules. This has been further confirmed by Malcolm O'Neill of the Complex Carbohydrate Research Center in Athens, Georgia. However, the relative configuration of hydroxy group at the fourth and fifth carbons has not been elucidated yet. From the kinetics of periodate oxidation (Fig. 3-3), all products seemed to have a trans configuration with respect to these two carbons, except that derived from D-glyceraldehyde 3-phosphate, which formed a chromogen rapidly destroyed in the oxidation procedure (Table 3-4).



Fig. 3-3. Kinetics of periodate oxidation of enzymatic products of spinach DS-Co with various substrates.

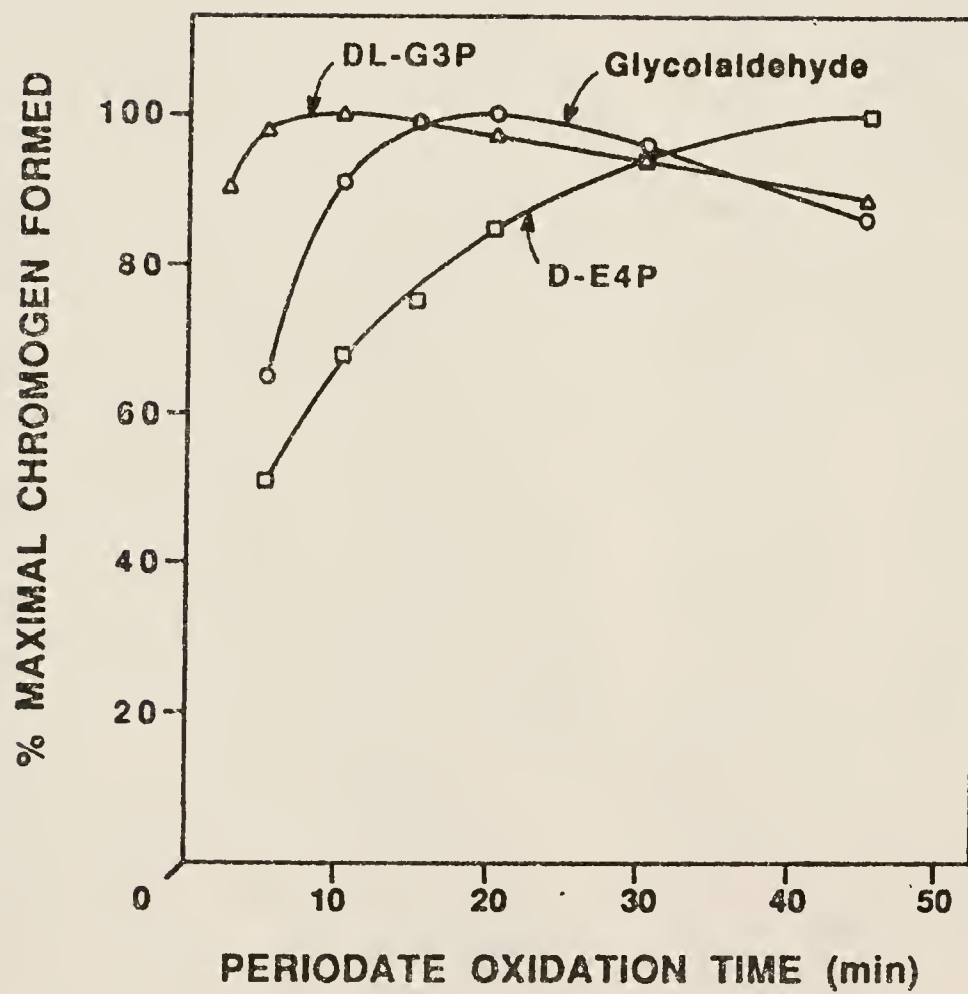


TABLE 3-3. Kinetic parameters for various substrates of spinach DS-Co

	Km (mM)	Vmax (nmol/min)	Vmax/Km
glycolaldehyde	8.6	20	2.32
glyoxylate	3.6	0.47	0.13
D-glyceraldehyde	3.5	5.0	1.43
L-glyceraldehyde	3.3	5.4	1.64
DL-glyceraldehyde- 3-phosphate	3.0	5.3	1.76
D-erythrose	2.5	2.2	0.88
L-erythrose	5.1	2.8	0.55
D-threose	8.4	5.2	0.62
L-threose	13.9	5.0	0.36
D-erythrose 4-phosphate	1.95	3.1	1.57

PEP at 3 mM was used in combination with various substrates under standard assay conditions.

### Regulation

The enzyme was not affected by phenylalanine, tyrosine, tryptophan, chorismate, prephenate, or aroenate when tested at 0.5 mM. Thioredoxin was not stimulatory.

TABLE 3-4. Relative configurations of hydroxy groups at C4 and C5 of enzymatic products of spinach DS-Co with various substrates as inferred from periodate oxidation kinetics

---

<u>Substrates</u>	<u>Configuration</u>
glycolaldehyde	trans
D-glyceraldehyde	trans
L-glyceraldehyde	trans
D-erythrose	trans
L-erythrose	trans
D-threose	trans
L-threose	trans
DL-glyceraldehyde-3-phosphate	cis
D-erythrose 4-phosphate	trans

---

### Discussion

Owing to its insensitivity to feedback inhibition by three aromatic amino acids and their intermediary metabolites, the role of DS-Co in aromatic amino acid biosynthesis has been a subject of interest. The evidence that this enzyme utilized many simple sugars other than E4P, the conventional substrate for DAHP synthesis, shed further light on its possible role in a hitherto unrecognized biochemical pathway. The possible implications of the enzyme in plant metabolism are discussed as follows.

### Metabolism of Two Carbon Compounds

It is still unknown whether glycolaldehyde is an intermediary metabolite in the biochemistry of plants. Since the report of glycolaldehyde dehydrogenase in 1960 by Davies, there has been no further investigation. Sources of glycolaldehyde could be decarboxylation of hydroxypyruvate, which in turn was derived from oxidation of glycerate or phosphoglycerate, or from the transamination of serine. The possibility that glycolaldehyde could be released from an active glycolaldehyde-enzyme complex during transfer reaction catalyzed by transketolase as reported in 1961 by Datta and Racker could also be considered.

Although the biogenesis of glycolaldehyde in the plant kingdom is still a mystery, 2-keto-3-deoxy-arabonate, the enzymatic product of DS-Co, originally isolated and characterized by Palleroni and Doudoroff in 1956, has been identified as an intermediary metabolite for the catabolism of arabinose to 2-ketoglutarate, and the following two catalytic steps, i.e., dehydration followed by dehydrogenation, have been documented in Pseudomonas saccharophila (Weimberg, 1959; Stoolmiller and Abeles, 1966). The source of  $\alpha$ -ketoglutarate required for transport into chloroplasts for reassimilation of ammonia released by photorespiration is unknown although its origin has been proposed to be within the mitochondria.

Glyoxylate, on the other hand, could be utilized by DS-Co to form a 4-hydroxy-2-oxoglutarate (4-HOG), which could also

generate  $\alpha$ -ketoglutarate in two enzyme steps. A recent report of an aldolase in *E. coli* which catalyzes the reversible formation of 4-HOG from pyruvate and glyoxylate supports this possibility (Patil and Dekker, 1990). Glyoxylate could be produced in vivo by means of (a) oxidation of glycolate from photorespiration (Ogren, 1984), (b) transamination of glycine (Harder and Quayle, 1971), (c) glyoxylate cycle by the activity of isocitrate lyase (Zelitch, 1988), and (d) degradation of allantoin (Thomas and Schrader, 1981). Hence, there exists a potential pathway for the replenishment of  $\alpha$ -ketoglutarate via the condensation of PEP and either glycolaldehyde or glyoxylate by DS-Co activity. These reactions are potentially significant in vivo because they could provide the cell with an alternative anaplerotic pathway to  $\alpha$ -ketoglutarate.

#### Metabolism of Three Carbon Compounds

The serendipitous discovery of D-glyceraldehyde 3-phosphate as a good substrate for DS-Co in higher plants has led to a hypothetical pathway where aromatic amino acid biosynthesis in cytosol may originate with the condensation of PEP with D-glyceraldehyde-3-P rather than D-erythrose-4-phosphate. This hypothetical phyto-shikimate pathway utilizes intermediates such as phyto-shikimate, phyto-chorismate, and phyto-prephenate molecules which differ from their conventional counterparts in replacement of the 1-carboxy substituent by a 1-hydroxy substituent. According to this

scheme, it is fortuitous that the cytosolic DS-Co and chorismate mutase-2 possess the substrate ambiguity to accept erythrose-4-P phosphate and chorismate in place of glyceraldehyde-3-P and phyto-chorismate, respectively. The much greater availability of triosephosphate than of erythrose-4-phosphate in the cytoplasm may accommodate the quantitatively great transit of carbon through the aromatic pathway to multiple connecting pathways of secondary metabolism.

2-Keto-3-deoxy-6-phosphogluconic acid was originally established as an intermediary metabolite of glucose metabolism in P. saccharophila in 1954 by MacGee and Doudoroff and its nonphosphorylated form, 2-keto-3-deoxygluconate, a possible product of DS-Co with D-glyceraldehyde-3-phosphate followed by dephosphorylation, has been reported to be a degradation product of gluconate by Aspergillus niger (Elzainy et al., 1973). On the other hand, 2-keto-3-deoxygalactonate and its phosphate ester were found to be a product of galactose metabolism in Gluconobacter liquefaciens (Stouthamer, 1961). Whether these groups of 2-keto-3-deoxy-sugar acids are involved in the hexose metabolism of higher plants has never been investigated.

#### Metabolism of Four Carbon Compounds

As a product of the oxidative pentose phosphate pathway, erythrose-4-phosphate may be an additional source of aromatic amino acid biosynthesis in the cytoplasm.



### Structural Component of Phytotoxin and Cell Wall

2-Keto-3-deoxygluconic acid, a possible enzymatic product of DS-Co with glyceraldehyde, was confirmed to be a component of a phytotoxic glycopeptide from potato plants infected with Corynebacterium sepedonicum (Strobel, 1970), and the carboxyl group of this acid was critical to the biological activity of the toxin (Johnson and Strobel, 1970). Although it is a good substrate for DS-Co, DL-glyceraldehyde has been demonstrated to be a potent inhibitor for the carbon fixation of spinach chloroplasts, and almost complete inhibition was observed at 10 mM concentration (Stokes and Walker, 1972). It was also shown to inhibit phosphoribulose kinase or phosphoribose isomerase (Bamberger and Avron, 1975), and the light activation of ribulose biphosphate carboxylase (Bahr and Jensen, 1977) in intact chloroplasts. On the other hand, glycolaldehyde was also reported to be more inhibitory than DL-glyceraldehyde on carbon assimilation of spinach chloroplasts (Sicher, 1984; Miller and Canvin, 1989). Furthermore, glyoxylate inhibition of ribulose biphosphate carboxylase was also observed in intact, lysed, and reconstituted chloroplasts (Campbell and Ogren, 1990). Under the auspices of DS-Co, PEP may serve as a sink for glyceraldehyde, glycolaldehyde, and glyoxylate for the synthesis of beneficial molecules when toxic molecules are inevitably produced in the plant cells, in addition to the central role it plays in plant metabolism (Davies, 1979).



During microbial infection the cell may convert products of glycolytic pathway to six carbon homologue of DAHP molecules due to increased respiration after infection (Kahl, 1974; Darvill and Albersheim, 1984).

2-Keto-3-deoxygalactonic acid has also been described as a constituent of an extracellular polysaccharide of Azotobacter vinelandii (Claus, 1965) and Vibrio parahaemolyticus (Kondo et al., 1989), its higher plant counterpart may await discovery as with the case of 2-keto-3-deoxy-octonate (York et al., 1985). In fact, 3-deoxy-D-lyxo-2-heptulosaric acid has also been identified as a cell wall component in higher plants and a green alga (Stevenson et al., 1988; Becker et al., 1989).

#### Multifunctionality

DS-Co may be a versatile enzyme that plays a different role at different physiological condition. Ambiguity of substrate utilization can be advantageous for the cell in situations where it is appropriate for a family of unrelated substrates to generate a family of related products. For example, acetolactate synthase condenses two pyruvate molecules or one pyruvate plus one ketobutyrate for the biosynthesis of valine and isoleucine respectively. Fractional product outputs of DS-Co may be dependent of developmental and environmental impacts. Compartmentation may also dictate the major catalytic reaction underway. For example, a form of DS-Co could be compartmented in glyoxysomes, where glyoxylate

formation is a major specialized function via the glyoxylate shunt. Thus, in castor bean seed tissue, glyoxylate may be a major source of  $\alpha$ -ketoglutarate via DS-Co, whereas E4P and/or G3P may be the source of cytoplasmic aromatic amino acids in other tissues via DS-Co.

## CHAPTER IV KDOP SYNTHASE

### Introduction

3-Deoxy-D-manno-octulosonate-8-phosphate (KDOP) synthase is an enzyme of lipopolysaccharide biosynthesis (Schmidt and Jann, 1983), thought until recently to be restricted to the purple-bacteria group of prokaryotes. Preliminary data showing the presence in potato tubers of KDOP synthase has been reported (Morris et al, 1989). These results are consistent with recent reports (York et al., 1985; Stevenson et al., 1988) of the presence of 3-deoxy-D-manno-octulosonate and related compounds in the cell walls of a variety of plants. Owing to substrate ambiguity, this KDOP synthase possessed weak activity as 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the initial catalytic step of aromatic amino acid biosynthesis. The plastid-localized DS-Mn and cytosol-localized DS-Co isozymes of DAHP synthase either did not catalyze the KDOP synthase reaction at all (DS-Mn), or did so very poorly (DS-Co). KDOP synthase from potato is demonstrated more directly after its fractionation free of DS-Mn and DS-Co. KDOP synthase from spinach was quite active and was selected for partial purification and further characterization. The general presence of KDOP synthase in crude extracts of a variety of higher plants was readily demonstrated.

## Materials and Methods

### Plant Material

Spinach, Idaho potatoes, broccoli, carrots, cucumbers, onions, sweet potatoes, apples, and cabbages were purchased from a local supermarket. Suspension-cultured cells of Nicotiana glauca (EE cells) that had been maintained continuously in exponential-phase growth (Bonner et al., 1988) were used. All plant material was washed with deionized water, frozen with liquid nitrogen, and ground to a fine powder by use of a Waring blender. The powders were stored at -70°C prior to extract preparation.

### Plant Extract Preparation and Enzyme Assays

All procedures were carried out at 0-4°C. A 45-g amount of powder of spinach or potato was mixed with 30 ml of buffer A (50 mM K phosphate, pH 7.2, containing 0.5%  $\beta$ -mercaptoethanol) and thawed at room temperature. The extract was clarified by centrifugation at 29,000 x g for 30 min. and filtered through Miracloth. A one-tenth volume of 2% protamine sulfate in buffer A was slowly added to the extract and stirred for 10 min.. The precipitate was removed by centrifugation at 29,000 x g for 20 min.

Spinach and potato extracts used for column chromatography were further treated as follows. The foregoing supernatants were brought to 70% (spinach) or to 60% (potato) of saturation with finely ground ammonium sulfate and stirred for 10 min. The protein precipitate was collected by

centrifugation at 29,000 x g for 20 min. and resuspended in a minimal volume of buffer B (10 mM EPPS, pH 7.5, and 50 mM KCl). Desalting was accomplished by passage through Sephadex G-25 (PD-10) columns equilibrated with buffer B according to the manufacturer's instructions. The eluate was used for the following chromatography.

#### DE-52 Column Chromatography of Plant Extracts

A 150-mg (84-mg for potato) of spinach protein was loaded onto a DE-52 cellulose column (1.5 x 19 cm) equilibrated with buffer B. The column was washed with 3 bed volumes of the buffer before a 400 ml gradient (50-300 mM KCl) in buffer B was applied. For the potato extracts a 400 ml gradient (50-500 mM KCl) was employed. The flow rate was 30 ml/hr, and fractions of 2.9 ml were collected. The enzyme assays for KDOP synthase, DS-Mn, and DS-Co were carried out as described previously (Morris et al, 1989).

#### Thin-layer Chromatography

The enzymatic product of KDOP synthase was spotted onto a cellulose-coated plastic plate (Chromagram of Eastman Kodak Company, Rochester, New York). Ascending chromatography was performed using an ethyl acetate/pyridine/acetic acid/water (4:5:1:4 by volume) mixture as the solvent. The chromatogram was dried and sprayed with reagent A (one volume of 0.1 M sodium periodate in water mixed with 9 volume of acetone) and after 15 min, with reagent B (One volume of ethylene glycol and 1/10 volume of 10 M sulfuric acid mixed with 19 volumes of

acetone. After 30 min the plate was sprayed with reagent C (2% of 2-thiobarbituric acid prepared in 96% hot ethanol) as described by Brade and Galanos (1983).

### Biochemicals

Sodium D-E4P, sodium D-ribose-5-P, sodium D-A5P, PEP (monocyclohexylammonium salt), D-arabinose, L-arabinose, D-ribose, 2-keto-3-deoxy-octonate (ammonium salt), protamine sulfate (from salmon), and buffers (EPPS, PIPES, MES, HEPES, BTP, CAPS, and Bicine) were obtained from Sigma (St. Louis, MO). DTT was purchased from Research Organics, (Cleveland, OH), and DE-52 anion exchanger was obtained from Whatman, Inc. (Clifton, NJ). PD-10 columns were from Pharmacia (Piscataway, NJ).

## Results

### KDOP Synthase in Potato

In previous work with potato, the elution profile of KDOP synthase from DEAE cellulose overlapped with that of the plastid-localized isozyme of DAHP synthase, DS-Mn. Under conditions where DTT (required by DS-Mn) and divalent cation (required by DS-Co) were omitted from reaction mixtures, a single minor peak of apparent DAHP synthase activity was discerned at an elution position between the DS-Mn and DS-Co isozymes. This enzyme proved to be much more active when E4P was replaced with A5P, and therefore appeared to be KDOP synthase.



In this study the conditions used for application of the salt gradient were modified to accomplish complete separation of DS-Mn and KDOP synthase (Fig. 2-1). The leading and peak fractions of KDOP synthase were also completely separated from DS-Co. KDOP synthase eluted at 0.11 M KCl.

#### KDOP Synthase in Spinach

Entirely comparable results were obtained following DEAE-cellulose chromatography of spinach extract (Fig. 2-2). DS-Mn eluted in the wash fractions, with KDOP synthase eluting earlier in the salt gradient than DS-Co. In spinach the relative amounts of DS-Mn and KDOP synthase exceeded those found in potato, while the relative amount of DS-Co was less. Given the better separation achieved and the greater activity of KDOP synthase in spinach, further characterization was carried out with the spinach enzyme.

Activity of KDOP synthase was followed as a function of incubation temperature (Fig. 4-1) in 20 min assays. A rather high temperature optimum of about 53°C was obtained. PEP was found to confer a striking degree of thermostability (Table 4-1). In the absence of PEP, KDOP synthase was unstable at even 27°C. This contrasts with the complete thermostability attained in the presence of PEP in the vicinity of 50°C. The pH optimum for activity (Fig. 4-2) was determined at both 37°C and 50°C. Activity increased sharply with pH up to about 6.2, and declined progressively (but to a modest extent) as pH was raised up to 10. Similar temperature and pH optima were found

for KDOP synthase from potato tuber and suspension-cultured cells of Nicotiana glauca (unpublished data). Divalent cations were not required for KDOP synthase activity (in contrast to DS-Co). KDOP synthase activity was also not stimulated by divalent cations (in contrast to DS-Mn) in experiments where 1 mM concentrations of  $Mg^{++}$ ,  $Mn^{++}$ ,  $Co^{++}$ ,  $Ni^{++}$ ,  $Fe^{++}$ ,  $Ca^{++}$ , and  $Ba^{++}$  were tested. Unlike the enzyme from Pseudomonas aeruginosa (Levine and Racker, 1959), KDOP synthase from spinach was not inhibited by 1 mM EDTA.

Partially purified spinach KDOP synthase was used to construct substrate saturation curves for A5P and PEP. Simple first-order kinetics were observed for A5P, and a double reciprocal replot gave a  $K_m$  value of 0.27 mM (Fig. 4-5). The affinity for PEP was so high that it was difficult to obtain accurate data points at very low PEP concentrations, especially without a continuous assay. The  $K_m$  value for PEP was estimated to be about 35  $\mu M$ . The substrate specificity of KDOP synthase was examined. PEP could not be replaced by pyruvate, thus ruling out the possibility that the enzyme might be a reversible aldolase. KDOP synthase was able to utilize E4P and R5P 24% and 7%, respectively, as well as D-arabinose-5-P when these substrates were compared at 3 mM concentrations. No activity was detected when D-arabinose, L-arabinose, or D-ribose were used as substrates in combination with PEP.



The product of KDOP synthase was identified as KDOP by means of thin-layer chromatography (Fig. 4-3). When crude extract was incubated with PEP and A5P, KDOP formed was rapidly dephosphorylated to KDO. Thus, a mixture of KDOP and KDO is visualized after 10 min of reaction in lane 2. The KDO migrated to the same position as did authentic KDO ( $R_f = 0.42$ ). The  $R_f$  value for KDOP was 0.21. In samples taken after elapsed reaction times of 30 minutes or greater, only KDO was visualized. On the other hand, partially purified enzyme recovered from anion-exchange chromatography (see Fig. 2-3) exhibited substantial separation from KDOP phosphatase activity. Thus, in lane 7, the majority of the reaction product was KDOP when partially purified enzyme was used, even after 7 hours of incubation of the reaction mixture.

Data shown in Fig. 4-4 indicate that on the basis of the kinetics of the oxidation by periodate of KDOP formed by KDOP synthase, the relative configuration of hydroxy groups at C-4 and C-5 of the molecule is cis. This orientation makes KDOP more prone to rapid oxidation by periodate than is DAHP, a molecule whose hydroxy groups at C-4 and C-5 exhibit a trans configuration (Ghalambor et al., 1966). The configuration of the eight-carbon product is hence inferred to be identical to that of bacterial KDOP.

As expected if KDOP synthase does not function as a DAHP synthase in vivo, aromatic amino acids, singly or in combination, did not feedback inhibit KDOP synthase.

Chorismate, prephenate, or arogenate (intermediary metabolites) having precedence as allosteric agents for DAHP synthase, also failed to inhibit the activity of KDOP synthase.

#### Other Plants

KDOP synthase can be assayed readily in crude extracts of higher plants. Table 4-2 shows the results of assays carried out with a variety of higher plants other than potato or spinach. Except for apple and cabbage, KDOP synthase activity was measured in all of the other plant species indicated. The result obtained showing that KDOP synthase levels in potato tissue assayed two days after mechanical wounding was not elevated contrasts with the elevated levels induced for both DS-Mn and DS-Co (Morris et al., 1989).

Table 4-1. Thermostability of spinach KDOP synthase

<u>Thermal treatment</u>	<u>% of control activity</u>	
	<u>+ PEP</u>	<u>- PEP</u>
27°C	100	82
37°C	105	72
47°C	102	62
57°C	92	37

Partially purified enzyme (Fig. 2-2) was incubated with or without 1.1 mM PEP at the indicated temperature for 30 min and removed to an ice bath prior to assay at 37°C under standard conditions.

Table 4-2. General presence of KDOP synthase in higher plants

Higher plant <sup>a</sup>	Specific activity <sup>b</sup>
Apple	0
Broccoli	0.12
Cabbage	0
Carrot	6.5
Cucumber	2.1
Onion	0.58
Sweet Potato	0.27
Potato, day 0 <sup>c</sup>	0.55
day 2 <sup>c</sup>	0.54
Spinach	1.7
Tobacco (EE cells) <sup>d</sup>	1.0

<sup>a</sup> Powders prepared as given in Methods were dissolved (3g/2ml) in Buffer A.

<sup>b</sup> nmol KDOP/min/mg protein.

<sup>c</sup> Extracts were prepared from the mechanical wounding experiment as described by Morris et al. (1989).

<sup>d</sup> Suspension-cultured cells of Nicotiana glauca continuously maintained in exponential-phase growth as described by Bonner et al. (1988).

Fig. 4-1. Temperature optimum of spinach KDOP synthase. Crude extract prepared as described in Methods was centrifuged at 29,000 x g and desalted on a Sephadex G-25 (PD-10) column equilibrated with 10 mM PIPES buffer (pH 7.2) containing 50 mM KCl. Reaction mixtures contained 100 mM BTP (pH 6.5), 3mM PEP, 3 mM A5P, and 12.5 mM KCl. Incubation duration was 20 min at the indicated temperatures.

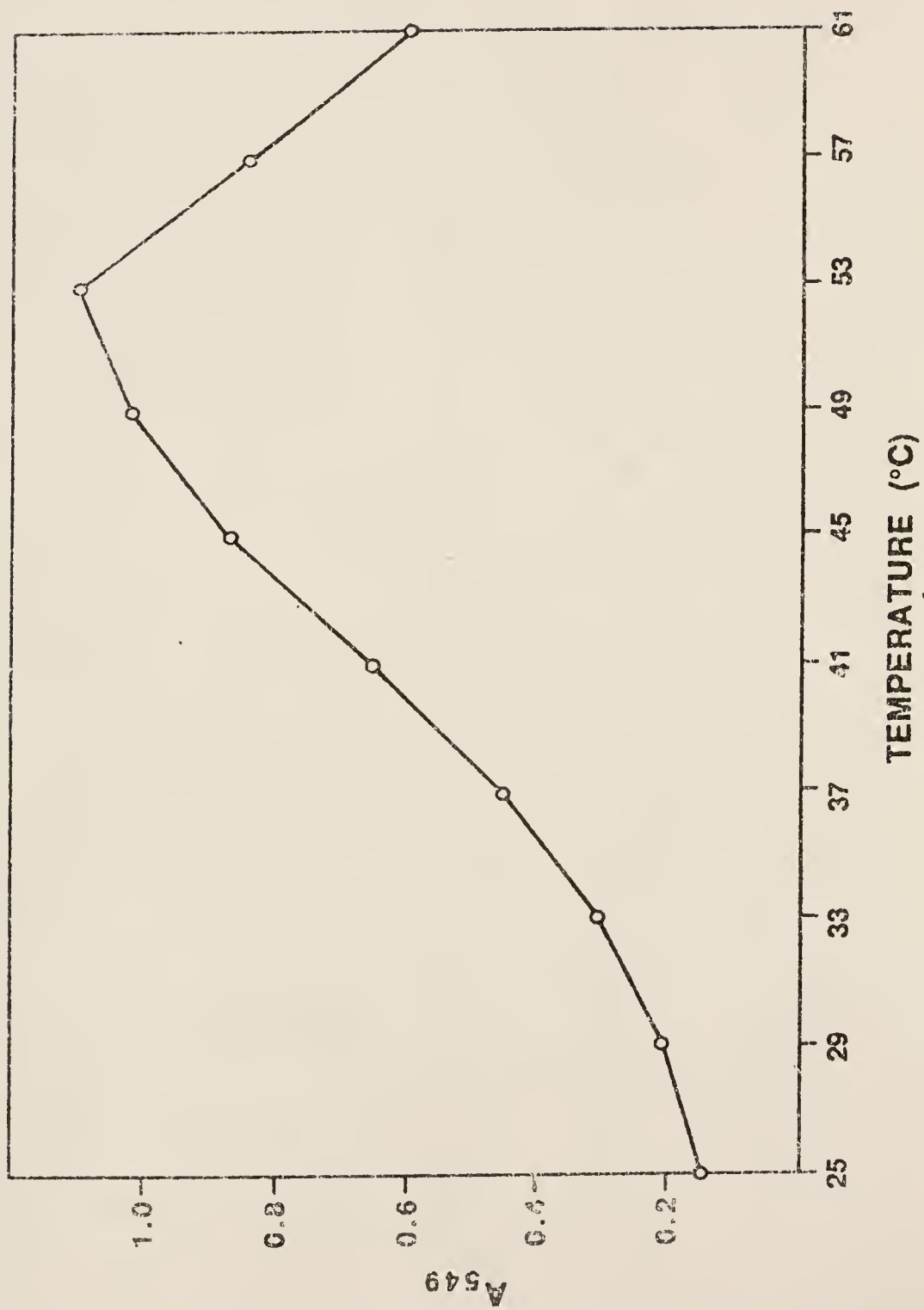
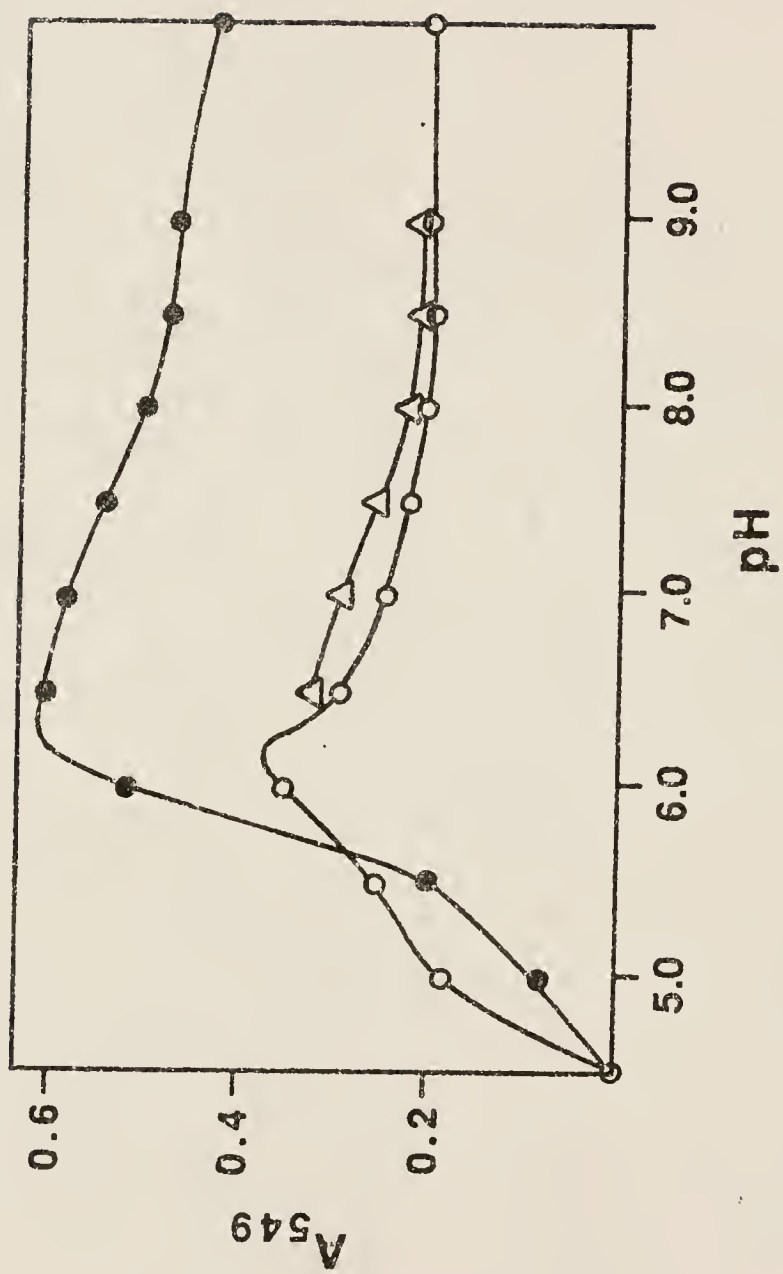


Fig. 4-2. pH optimum of KDOP synthase from spinach. The enzyme preparation was the same as described in the legend of Fig. 4-1. Buffers for enzyme assay were made 50 mM with citrate (pH 4.5), malate (pH 5.0), citrate (pH 5.5), MES (pH 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5), EPPS (pH 8.0), Bicine (pH 8.5), BTP (pH 9.0), and CAPS (pH 10.0). Symbols: o-o, assay at 50°C for 10 min. using above-cited buffers at a given pH; o-o, assay at 37°C for 20 min using above cited buffers at a given pH; , assay at 37°C for 20 min with BTP buffer adjusted to the indicated pH values.





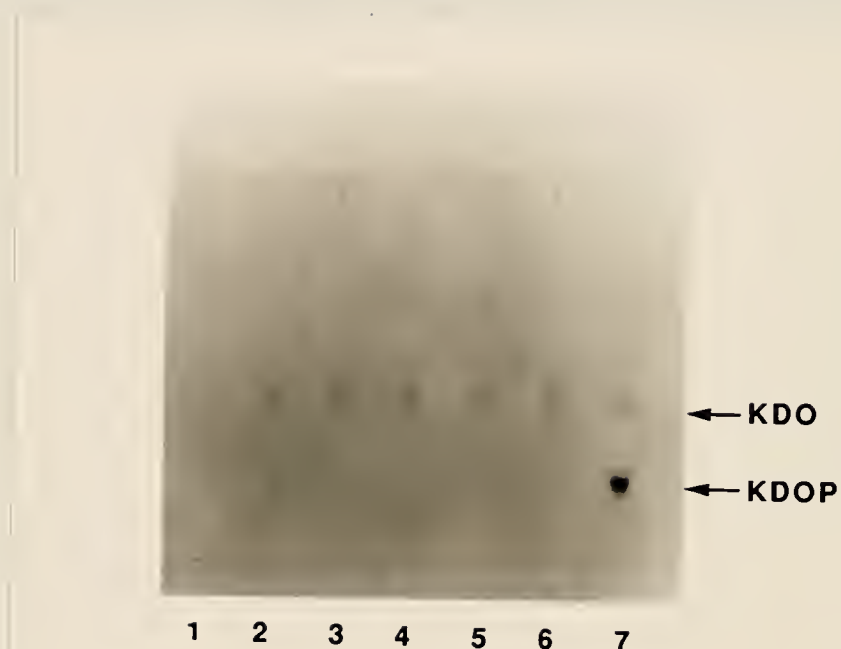


Fig. 4-3. Thin-layer chromatography of KDOP and KDO. Lanes 1 to 6 were spotted with enzyme assay aliquots ( $5\ \mu\text{l}$ ) obtained after 0, 10, 30, 60, 120, and 420 min of reaction time, using desalted crude extracts from spinach. Lane 7 was spotted with a 7-hr reaction mixture obtained by use of partially purified KDOP synthase from spinach (Fig. 2-2).

Fig. 4-4. Kinetics of oxidation of KDOP by periodate. KDOP was formed as the reaction product of KDOP synthase, and DAHP was formed as the reaction product of DS-Co in 1.6 ml reaction mixtures. Authentic KDO was purchased from Sigma. The enzyme reactions were stopped with 0.4 ml of 20% TCA and protein was removed. Periodate oxidation was terminated with excess arsenite at the times indicated.

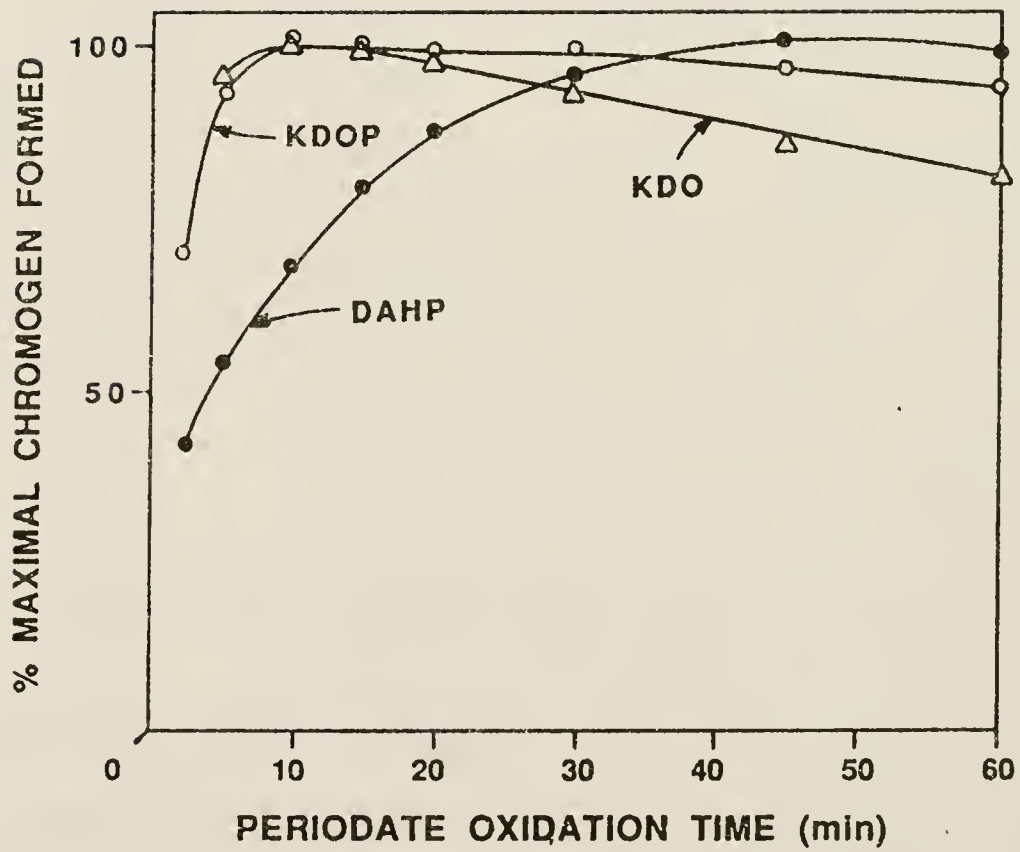
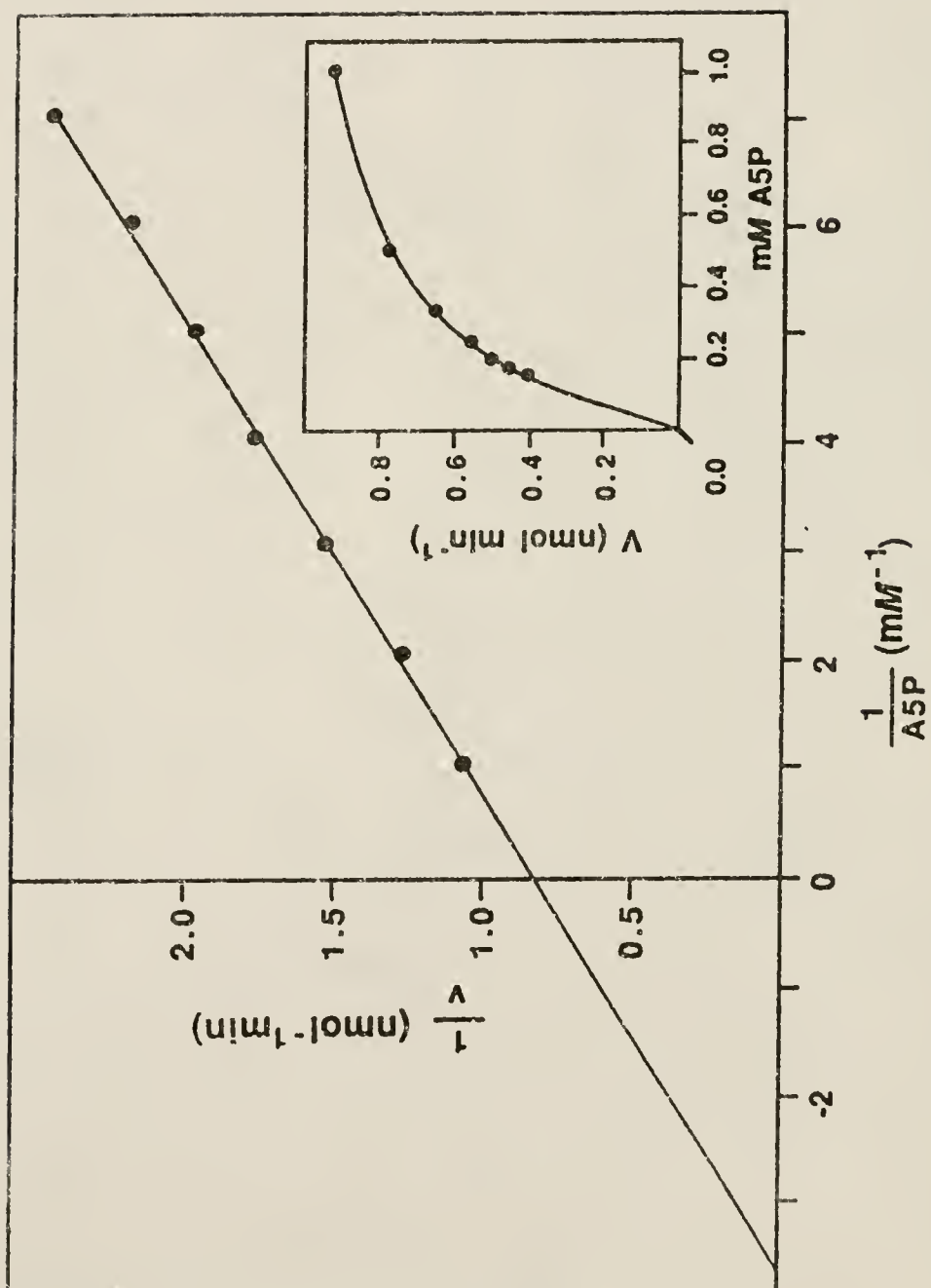


Fig. 4-5. Double reciprocal plot for arabinose-5-phosphate. PEP was held at 3 mM concentration for the assays.



### Discussion

Our initial finding of KDOP synthase in potato (Morris et al., 1989) was unexpected because this enzyme has only been described in gram-negative prokaryotes. However, with the appreciation that KDO residues have been reported to be present in the rhamno-galacturonan-II pectic polysaccharide of a wide variety of plant cell walls (York et al., 1985; Stevenson et al., 1988), the enzymatic formation of KDOP by higher plants is not surprising. At this time a specific functional role of KDOP in plant cell-wall architecture analogous to its well-defined role in microbial lipopolysaccharides is completely unknown.

KDOP synthase from spinach and potato was found to exhibit substrate ambiguity, accepting E4P 24% as well as A5P under conditions of substrate saturation. The cytosolic DAHP synthase isozyme (DS-Co) also exhibits substrate ambiguity, accepting A5P 8% as well as E4P under conditions of substrate saturation. Such a degree of substrate ambiguity has not been reported before for these enzymes from any source. In fact, three isozymes of DAHP synthase were detected in pea by Rothe et al. (1976), and in carrot by Suzich et al. (1985). One of these activities might be KDOP synthase in view of the results obtained in our investigation.

## REFERENCES

- Bahr, J. T. and Jensen, R. G. 1978. Activation of Ribulose Bisphosphate Carboxylase by CO<sub>2</sub> and Light. Arch. Biochem. Biophys. 185: 39-48.
- Bamberger, E. S. and Avron, M. 1975. Site of Action of Inhibitors of Carbon Dioxide Assimilation by Whole Lettuce Chloroplasts. Plant Physiol. 56: 481-485.
- Becker, B., Hard, K., Melkonian, M., Kamerling, J. P., and Vliegenthart, J. F. G. 1989. Identification of 3-Deoxy-manno-2-Octulosonic Acid, 3-Deoxy-5-O-Metyl-manno-2-Octulosonic Acid and 3-Deoxy-lyxo-2-Heptulosaric Acid in the Cell Wall (Theca) of the Green Alga Tetraselmis striata Butcher (Prasinophyceae). Eur. J. Biochem. 182: 153-160.
- Bonner, C. A., Kenyon, C., and Jensen, R. A. 1988. Physiological and Biochemical Characterization of a Suspension Culture System for Sustained Exponential Growth of Nicotiana glauca. Physiol. Plant. 74: 1-10.
- Brade, H. and Galanos, C. 1983. A Method to Detect 2-Keto-3-Deoxyoctanate and Related Compounds on Pherograms and Chromatograms. Anal. Biochem. 132: 158-159.
- Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72: 248-254.
- Buchanan, B. B. 1980. Role of Light in the Regulation of Chloroplast Enzymes. Ann. Rev. Plant Physiol. 31: 341-374.
- Byng, G. S., Whitaker, R. J., Shapiro, C. L., and Jensen, A. R. 1981. The Aromatic Amino Acid Pathway Branches at L-Arogenate in Euglena gracilis. Mol. Cell Biol. 1: 426-438.
- Campbell, W. J. and Ogren, W. L. 1990. Glyoxylate Inhibition of Ribulosebisphosphate Carboxylase/Oxygenase Activation in Intact, Lysed, and Reconstituted Chloroplasts. Photosynth. Res. 23: 257-268.

- Claus, D. 1965. 2-Keto-3-Deoxygalactonic Acid as a Constituent of an Extracellular Polysaccharide of Azotobacter vinelandii. Biochem. Biophys. Res. Commun. 20: 745-751.
- Crawford, N. A., Yee, B. C., Hutcheson, S. W., Wolosiuk, R. A., and Buchanan B. B. 1986. Enzyme Regulation in C<sub>4</sub> Photosynthesis: Purification, Properties, and Activities of Thioredoxins from C<sub>4</sub> and C<sub>3</sub> Plants. Arch. Biochem. Biophys. 244: 1-15
- Darvill, A. G. and Albersheim, A. 1984. Phytoalexins and Their Elicitors. Ann. Rev. Plant Physiol. 35: 243-75.
- Datta, A. G. and Racker, E. 1961. Mechanism of Action of Transketolase. J. Biol. Chem. 236: 624-628.
- Davies, D. D. 1960. The Purification and Properties of Glycolaldehyde Dehydrogenase. J. Exp. Bot. 11: 289-295.
- Davies, D. D. 1979. The Central Role of Phosphoenolpyruvate in Plant Metabolism. Ann. Rev. Plant Physiol. 30: 131-158.
- Dyer, W. E., Henstrand, J. M., Handa, A. K., and Herrmann, K. M. 1989. Wounding Induces the First Enzyme of the Shikimate Pathway in Solanaceae. Proc. Natl. Acad. Sci. USA. 86: 7370-7373.
- Dyer, W. E., Weaver, L. M., Zhao, J., Kuhn, D. N., Weller, S. C., and Herrmann, K. M. 1990. A cDNA Encoding 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase from Solanum tuberosum L. J. Biol. Chem. 265: 1608-1614.
- Dyer, W. E., Weller, S. C., Bressan, R. A., and Herrmann, K. M. 1988. Glyphosate Tolerance in Tobacco (Nicotiana tabacum L.) Plant Physiol. 88: 661-666.
- Elzainy, T. A., Hassan, M. M., and Allam, A. M. 1973. New Pathway for Nonphosphorylated Degradation of Gluconate by Aspergillus niger. J. Bacteriol. 114: 457-459.
- Gaines, C. G., Byng, G. S., Whitaker, R. J., and Jensen R. A. 1982. L-Tyrosine Regulation and Biosynthesis via Arogenate Dehydrogenase in Suspension-Cultured Cells of Nicotiana glauca L. Sp. et Comes. Planta. 156: 233-240.
- Ganson, R. J., D'Amato, T. A., and Jensen, R. A. 1986. The Two-Isozyme System of 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase in Nicotiana glauca L. and Other Higher Plants. Plant Physiol. 82: 203-210.



- Ganson, R. J. and Jensen, R. A. 1987. Response of Cytosolic-isozyme and Plastid-Isozyme Levels of 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase to Physiological State of Nicotiana glauca in Suspension Culture. *Plant Physiol.* 83: 479-482.
- Ghalambor, M. A., Levine, E. M., and Heath, E. C. 1966. The Biosynthesis of Cell Wall Lipopolysaccharide in Escherichia coli. *J. Biol. Chem.* 241: 3207-3215.
- Goers, S. K. and Jensen, R. A. 1984. The Differential Allosteric Regulation of Two Chorismate-Mutase Isoenzymes of Nicotiana glauca. *Planta.* 162: 117-124.
- Graziana, A. and Boudet, A. M. 1980. 3-Deoxy-D-arabino Heptulosonate 7-Phosphate Synthase from Zea mays: General Properties and Regulation by Tryptophan. *Plant and Cell Physiol.* 21: 793-802.
- Harder, W. and Quayle, J. R. 1971. The Biosynthesis of Serine and Glycine in Pseudomonas AM 1 with Special Reference to Growth on Carbon Sources Other Than C<sub>4</sub> Compounds. *Biochem. J.* 121: 753-762.
- Huisman, O. C. and Kosuge, T. 1974. Regulation of Aromatic Amino Acid Biosynthesis in Higher Plants. *J. Biol. Chem.* 249: 6842-6848.
- Jacquot, J. P., Gadgil, P., Nishizawa, A. N., Yee, B. C., Crawford, N. A., and Buchanan, B. B. 1984. Enzyme Regulation in C<sub>4</sub> Photosynthesis: Mechanism of Activation of NADP-Malate Dehydrogenase by Reduced Thioredoxin. *Arch. Biochem. Biophys.* 228: 170-178.
- Jensen, R. A. 1985. The Shikimate/Arogenate Pathway: Link Between Carbohydrate Metabolism and Secondary Metabolism. *Physiol. Plant.* 66: 164-168.
- Jensen, R. A. 1986. Tyrosine and Phenylalanine Biosynthesis: Relationship Between Alternative Pathways, Regulation and Subcellular Location. *Rec. Adv. Phytochem.* 20: 57-82.
- Jensen, R. A., Morris, P. F., Bonner, C. A., and Zamir, L. O. 1989. Biochemical Interface Between Aromatic Amino Acid Biosynthesis and Secondary Metabolism. in *Biogenesis and Biodegradation of Plant Cell Wall Polymers*, eds. Lewis, N. G. and Paice, M. G. (Amer. Chem. Soc. Symp. Series) 399: 89-107.
- Jensen, R. A. and Nester, E. W. 1966. Regulatory Enzymes of Aromatic Amino Acid Biosynthesis in Bacillus subtilis. *J. Biol. Chem.* 241: 3373-3380.

- Johnson, T. B. and Strobel, G. A. 1970. The Active Site on the Phytotoxin of Corynebacterium sepedonicum. Plant Physiol. 45: 761-764.
- Jung, E., Zamir, L. O., and Jensen, R. A. 1986. Chloroplasts of Higher Plants Synthesize L-Phenylalanine via L-Aroenate. Proc. Natl. Acad. Sci. USA. 83: 7231-7235.
- Kahl, G. 1974. Metabolism in Plant Tissue Slices. Bot. Rev. 40: 263-314.
- Kondo, S., Zahringer, U., Rietschel, E. T., and Hisatsune, K. 1989. Isolation and Identification of 3-Deoxy-D-threo-Hexulosonic Acid as a Constituent of the Lipopolysaccharide of Vibrio parahaemolyticus Serotypes 07 and 012. Carbohydr. Res. 188: 97-104.
- Levin, D. H. and Racker, E. 1959. Condensation of Arabinose 5-Phosphate and Phosphorylenol Pyruvate by 2-Keto-3-Deoxy-8-Phosphooctonic Acid Synthetase. J. Biol. Chem. 234: 2532-2539.
- MacGee, J. and Doudoroff, M. 1954. A New Phosphorylated Intermediate in Glucose Oxidation. J. Biol. Chem. 210: 617.
- McCue, K. F. and Conn, E. E. 1989. Induction of 3-Deoxy-D-arabino-Heptulosonate-7-Phosphate Synthase Activity by Fungal Elicitor in Cultures of Petroselinum crispum. Proc. Natl. Acad. Sci. USA. 86: 7374-7377.
- Miller, A. G. and Canvin, D. T. 1989. Glycolaldehyde Inhibits CO<sub>2</sub> Fixation in the Cyanobacterium Synechococcus UTEX 625 Without Inhibiting the Accumulation of Inorganic Carbon or the Associated Quenching of Chlorophyll a Fluorescence. Plant Physiol. 91: 1044-1049.
- Minamikawa, T. 1967. A Study on 3-Deoxy-D-arabino-Heptulosonic Acid 7-Phosphate Synthase in Higher Plants. Plant and Cell Physiol. 8: 695-707.
- Minamikawa, T. and Uritani, I. 1967. 3-Deoxy-D-arabino-Heptulosonic Acid 7-Phosphate Synthase in Sweet Potato Roots. J. Biochem. 61: 367-372.
- Morris, P. F., Bonner, C. A., Doong, R. L., Subramaniam, P., Fischer, R. S., and Jensen, R. A. 1989. Mechanical Wounding Induces the Entire Primary Pathway of Aromatic Amino Acid Biosynthesis. Plant Physiol. (Life Sci. Advances, Series B) 8:35-39.

- Morris, P. F., Doong, R. L., and Jensen, R. A. 1989. Evidence from Solanum tuberosum in Support of the Dual-Pathway Hypothesis of Aromatic Biosynthesis. *Plant Physiol.* 89: 10-14.
- Ogren, W. L. 1984. Photorespiration: Pathways, Regulation, and Modification. *Ann. Rev. Plant Physiol.* 35: 415-42.
- Palleroni, N. J. and Doudoroff, M. 1956. Characterization and Properties of 2-Keto-3-Deoxy-D-Arabonic Acid. *J. Biol. Chem.* 223: 499.
- Patil, R. V. and Dekker, E. E. 1990. Cloning and Expression of 2-Keto-4-Hydroxyglutarate (KHG) Aldolase of Escherichia coli. Abstract. The FASEB Journal. 4: A1952 #1507 (New Orleans).
- Pinto, J. E. B. P., Dyer, W. E., Weller, S. C., and Herrmann, K. M. 1988. Glyphosate induces 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthase in Potato (Solanum tuberosum L.) Cells Grown in Suspension Culture. *Plant Physiol.* 87: 891-893.
- Pinto, J. E. B. P., Suzich, J. A., and Herrmann, K. M. 1986. 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase from Potato Tuber (Solanum tuberosum L.). *Plant Physiol.* 82: 1040-1044.
- Reinink, M. and Borstlap, A. C. 1982. 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase from Pea Leaves: Inhibition by L-Tyrosine. *Plant Science Letters.* 26: 167-171.
- Richardson, K. E., Miedema, E., and Vondor, S. L. The Oxidation of Ethanolamine by Plant Tissue Preparations. *Canadian J. Biochem.* 44: 1271-1275.
- Rothe, G. M., Maurer, W., and Mielke, C. 1976. A Study on 3-Deoxy-D-arabino-Heptulosonic Acid 7-Phosphate Synthase in Higher Plants. *Ber. Deutsch. Bot. Ges. Bd.* 89: 163-173.
- Rubin, J. L. and Jensen, R. A. 1985. Differentially Regulated Isozymes of 3-Deoxy-D-arabino-Heptulosonate-7-Phosphate Synthase from Seedlings of Vigna radiata [L.] Wilczek. *Plant Physiol.* 79: 711-718.
- Sacher, J. A., Towers, G. H. N., and Davies, D. D. 1972. Effect of Light and Aging on Enzymes, Particularly Phenylalanine Ammonia Lyase, in Discs of Storage Tissue. *Phytochemistry.* 11: 2383-2391.

- Schmidt, C. L., Danneel, H.-J., Schultz, G., and Buchanan, B. B. 1990. Shikimate Kinase from Spinach Chloroplasts. *Plant Physiol.* 93: 758-766.
- Schmidt, M. A. and Jann, K. 1983. Structure of the 2-Keto-3-Deoxy-D-manno-Octonic Acid Containing Capsular Polysaccharide (K 12 Antigen) of the Urinary Tract Infective Escherichia coli 04: K 12: H<sup>-</sup>. *Eur. J. Biochem.* 131: 509-517.
- Schmidt, C. L. and Schultz, G. 1987. Stimulation by Thioredoxin of Shikimate Kinase from Spinach Chloroplasts. *Physiol. Plantarum.* 70: 65-67.
- Sicher, R. C. 1984. Glycolaldehyde Inhibition of Photosynthetic Carbon Assimilation by Isolated Chloroplasts and Protoplasts. In C. Sybesma. ed, *Advances in Photosynthetic Research*, Vol III. Martinez Nijhoff/Dr. W. Junk, The Hague, pp 413-416.
- Srinivasan, P. R. and Sprinson, D. B. 1959. 2-Keto-3-Deoxy-D-arabo-Heptonic Acid 7-Phosphate Synthetase. *J. Biol. Chem.* 234: 716-722.
- Stevenson, T. T., Darvill, A. G., and Albersheim, P. 1988. 3-Deoxy-D-lyxo-2-Heptulosaric Acid, a Component of the Plant Cell-Wall Polysaccharide Rhamnogalacturonan-II. *Carbohydr. Res.* 179: 269-288.
- Stokes, D. M. and Walker, D. A. 1972. Photosynthesis by Isolated Chloroplasts: Inhibition by DL-Glyceraldehyde of Carbon Dioxide Assimilation. *Biochem. J.* 128: 1147-1157.
- Stoolmiller, A. C. and Abeles, R. H. 1966. Formation of  $\alpha$ -Ketoglutaric Semialdehyde from L-2-Keto-3-Deoxyarabonic Acid and Isolation of L-2-Keto-3-Deoxyarabonate Dehydratase from Pseudomonas saccharophila. *J. Biol. Chem.* 241: 5764-5771.
- Stouthamer, A. H. 1961. Glucose and Galactose Metabolism in Gluconobacter liquefaciens. *Biochim. Biophys. Acta.* 48: 484-500.
- Strobel, G. A. 1970. A Phytotoxic Glycopeptide from Potato Plants Infected with Corynebacterium sepedonicum. *J. Biol. Chem.* 245: 32-38.
- Suzich, J. A., Dean, J. F. D., and Herrmann, K. M. 1985. 3-Deoxy-D-arabino-Heptulosonate 7-Phosphate Synthase from Carrot Root (Daucus carota) is a Hysteretic Enzyme. *Plant Physiol.* 79: 765-770.




- Suzich, J. A., Ranjeva, R., Hasegawa, P. M., and Herrmann, K. M. 1984. Regulation of the Shikimate Pathway of Carrot Cells in Suspension Culture. *Plant Physiol.* 75: 369-371.
- Thomas, R. J. and Schrader, L. E. 1981. Ureide Metabolism in Higher Plants. *Phytochemistry.* 20: 361-371.
- Weber, H. L. and Bock, A. 1968. Comparative Studies on the Regulation of DAHP Synthase Activity in Blue-Green and Green algae. *Archives for Microbiology.* 61: 159-168.
- Weimberg, R. 1959. L-2-Keto-4,5-Dihydroxyvaleric Acid: an Intermediate in the Oxidation of L-Arabinose by Pseudomonas saccharophila. *J. Biol. Chem.* 234: 727.
- Weissbach, A. and Hurwitz, J. 1959. The Formation of 2-Keto-3-Deoxyheptonic Acid in Extracts of Escherichia coli B. *J. Biol. Chem.* 234: 705-709.
- Wolosiuk, R. A., Crawford, N. A., Yee, B. C., and Buchanan, B. B. 1979. Isolation of Three Thioredoxins from Spinach Leaves. *J. Biol. Chem.* 254: 1627-1632.
- York, W. S., Darvill, A. G., McNeil, M., and Albersheim, P. 1985. 3-Deoxy-D-manno-2-Octulosonic Acid (KDO) is a Component of Rhamnogalacturonan II, a Pectic Polysaccharide in the Primary Cell Walls of Plants. *Carbohydr. Res.* 138: 109-126.
- Zelitch, I. 1988. Synthesis of Glycolate from Pyruvate via Isocitrate Lyase by Tobacco Leaves in Light. *Plant Physiol.* 86: 463-468.
- Zucker, M. 1965. Induction of Phenylalanine Deaminase by Light and Its Relation to Chlorogenic Acid Synthesis in Potato Tuber Tissue. *Plant Physiol.* 40: 779-784.
- Zucker, M. 1968. Sequential Induction of Phenylalanine Ammonia-Lyase and a Lyase-Inactivating System in Potato Tuber Disks. *Plant Physiol.* 43: 365-374.

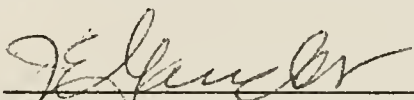
## BIOGRAPHICAL SKETCH

Ron Lou Doong (previously known as Rong-Luh Doong) was born on March 28, 1949 in Tainan, Taiwan, Republic of China. He received his B.S. degree in 1971 and M.S. degree in plant pathology in 1975 from National Taiwan University. After working for Taiwan Banana Research Institute, he immigrated to the United States of America and became a citizen in 1989. He is currently a candidate for a Ph.D. degree in the Department of Microbiology and Cell Science.

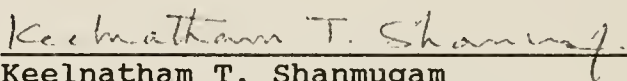
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
\_\_\_\_\_  
Roy A. Jensen, Chairman  
Professor of Microbiology and  
Cell Science

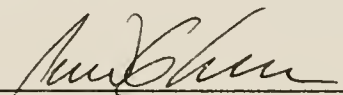
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
\_\_\_\_\_  
John E. Gander  
Professor of Microbiology and  
Cell Science

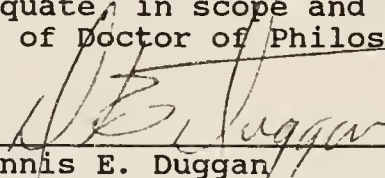
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
\_\_\_\_\_  
Keelnatham T. Shanmugam  
Professor of Microbiology and  
Cell Science

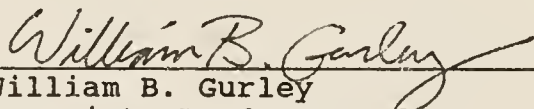
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
\_\_\_\_\_  
Paul Chun  
Professor of Biochemistry and  
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

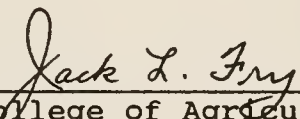
  
Dennis E. Duggan  
Associate Professor of  
Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
William B. Gurley  
Associate Professor of  
Microbiology and Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1990

  
Dean, College of Agriculture

\_\_\_\_\_  
Dean, Graduate School



UNIVERSITY OF FLORIDA



3 1262 08553 8154